AN EVALUATION OF IMMUNOLOGICALLY SPECIFIC INHIBITION OF CARDIAC ALLOGRAFT REJECTION IN RATS

Submitted by

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I am indebted to Professor Sir Michael Woodruff for his advice and help in all matters relating to this study. Before joining his department I knew little immunology but through his encouragement the subject has come to hold the greatest fascination for me.

My enthusiasm has also been sustained by the generous interest shown by my colleagues. Dr. Keith James has consistently encouraged and advised me, and was kind enough to carry out fractionation of some serum samples. Dr. Rodger Dalton gave valuable advice about the planning of this thesis and helped with the statistics. Dr. Norman Anderson steadfastly carried out thoracic duct cannulations to provide me with lymphocytes. Dr. John Sanders performed the microcytotoxicity tests the results of which are included in Chapter IX.

I have also been lucky to receive valuable technical help with a variety of problems from Mr. Frank Cochrane. I am also grateful to Mr. Tom Wilson who in the early stages of the project gave help with the technique of heart transplantation in the rat. Mrs. Sandra Bouch and Mrs. Margot Reid have given expert technical

assistance without which the experiments could not have been carried out. Throughout the study Mr. Stuart Robertson provided me with expertly prepared histological sections of rejected hearts.

I also owe thanks to the British Heart Foundation whose generous grant supported this project which was undertaken in the Department of Surgery, University of Edinburgh.

Lastly I would like to thank Miss Linda Law for her patience and skill in typing this thesis.

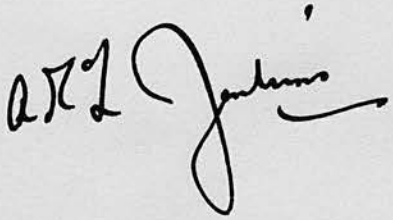
DECLARATION

The work presented in this thesis represents a summary of studies carried out by myself while working in the Department of Surgery under the direction of Sir Michael Woodruff. The project was supported by a grant from the British Heart Foundation.

In 1969 I joined the research staff of the department. I was encouraged by Sir Michael to perfect the technique of heterotopic heart transplantation in the rat for the purpose of immunological studies. Throughout the project I have been responsible for performing all of the heart transplant operations which number over 500.

Following perfection of the technique Sir Michael gave guidance in the direction of the work and I was responsible for the actual performance of the experiments. I received technical help from Dr. Norman Anderson who carried out thoracic duct cannulations and from Dr. Keith James and Dr. John Sanders who assisted with antibody studies in the rat.

I alone am responsible for the composition of this thesis.



SUMMARY

The literature in relation to immunological enhancement is reviewed. The development and technique of heart transplantation in the rat is described and the value of this experimental model is assessed.

Studies are presented showing that intravenous pre-treatment of cardiac allograft recipients with donor strain blood induced marked prolongation of graft survival. Skin allograft survival was slightly prolonged by similar means. Using blood from rats of a strain which differed at the major histocompatibility locus from either the graft donor or recipient the effect was shown to be immunologically specific. Pre-treatment with thoracic duct lymphocytes and with spleen cells of donor strain induced prolongation of cardiac allograft survival similar to that following injection of whole blood. Administration of donor strain erythrocytes and plasma did not affect graft survival.

Using donor strain spleen cells the optimum conditions of pre-treatment were assessed. 10^7 cells injected intravenously two weeks prior to transplantation produced the maximum increase in graft survival. The

same number of cells given as late as during the transplant operation, though less effective, still resulted in a highly significant prolongation of graft survival. Cells given on the day after transplantation were without effect.

The prolonged survival of cardiac allografts in pre-treated rats was not reduced by injection of further donor strain blood or of sensitised recipient strain spleen cells given two to three weeks after transplantation.

Antibody studies were performed on rats pre-treated with donor strain blood, on rats receiving cardiac allografts, and on pre-treated rats which subsequently received cardiac allografts. Injection of donor strain blood induced high titres of cytotoxic antibody one week after injection. This was shown to be mostly of 19S class. At two weeks this had subsided and was succeeded by a more prolonged response which at two weeks was shown to be mainly of 7S class. The titres of this second response were raised following transplantation of a cardiac allograft two weeks after blood pre-treatment.

Passive transfer of serum from rats injected with donor strain blood did not induce prolongation of graft survival in otherwise untreated recipients. Similar

serum perfused through the coronary circulation of isolated hearts immediately before transplantation also did not affect graft survival time.

Using F_1 hybrid animals the reproducibility of the graft-versus-host assay of Ford, Burr and Simonsen (1970) was tested. This assay was used to evaluate the immunocompetence of thoracic duct lymphocytes of rats pre-treated with donor strain blood. Such cells showed normal graft-versus-host reactivity. As a further test of immunocompetence pre-treated rats bearing long surviving cardiac allografts received skin grafts of donor strain. Such grafts were rejected in the time characteristic of control skin grafts. These results show that the pre-treatment schedule used in these studies did not induce a state of immunological tolerance in the sense which was implied by Billingham, Brent and Medawar (1953).

The final experiment was designed to assess possible alteration of antigenicity of long surviving cardiac allografts borne by pre-treated rats. The hearts were retransplanted into untreated rats of donor and recipient strain. In the former indefinite graft survival occurred. In rats of recipient strain the survival of

a proportion of retransplanted hearts was prolonged.

The possible role of immunological enhancement in the experiments is discussed.

It is concluded that the phenomenon may be of potential value in clinical transplantation, but owing to species differences experimental results obtained with rats are not necessarily directly applicable to man.

STATISTICAL ANALYSIS AND PRESENTATION OF RESULTS

In the presentation of the experimental results in this thesis the following mathematical and statistical techniques have been applied (Snedicor, 1956).

Arithmetical means are shown together with standard errors. Geometric means are followed by numbers in parenthesis which show the effect of adding or subtracting 1 standard error of the log mean. Comparison between means have been made using Student's t Test. The probabilities cited with this test are designated N.S. when not significant. Otherwise they are expressed as 0.05 to 0.001. Only those probabilities greater than 0.05 (i.e. 5%) are considered to be biologically significant.

INTRODUCTION

INTRODUCTION

Organ transplantation provides a unique approach to human illness in which failure of, or damage to, a vital organ may jeopardize the life of the individual. The potential of transplant surgery has been recognised increasingly in recent years, and this has led to resolution of many of the purely technical problems. The introduction of extracorporeal circulation with cardiac bypass brought within the bounds of possibility replacement of the heart itself and surgical techniques have now proved this to be feasible. Griepp, Stinson, Dong, Clark and Shumway (1971) reported a series of 26 such transplants, and more than a quarter of the patients were alive at the end of one year. Kidney transplants have now become routine and the 8th Report of the Human Kidney Transplant Registry (1971) records data from a total of 3,645 transplants. Experimentation with transplants of other organs is proceeding. However although technical problems associated with transplantation are being steadily resolved, the solution to rejection of allografts remains.

The phenomenon of rejection was reviewed by Loeb in

1930. The characteristic features of round cell infiltration, vascular stasis, oedema and necrosis of grafts are now familiar to all concerned with transplant surgery. The success of grafts exchanged between identical twins underlines the immunological basis of rejection of grafts between unrelated individuals.

The search for methods of preventing rejection of allografts has led to a diversity of approaches. One is aimed at circumventing the problem by matching the tissue types of donor and recipient and has been reviewed by Joysey (1971). This concept promoted the formation of the organisation 'Eurotransplant' which types and distributes organs to centres having the most closely compatible recipients available. Some success has been claimed for the scheme with regard to improved survival of renal transplants (van Rood, van Leeuwen, Pearce and van der Does, 1969). The complexity of the Human Leucocyte Antigen (HL-A) system however makes perfect matching extremely difficult to obtain in practice. Kissmeyer-Nielsen, Svejgaard and Hauge (1969) calculated that 1008 different HL-A phenotypes are possible, and that a random chance of finding two unrelated individuals with identical tissue types is

approximately 1 in 1000. A very large recipient pool is necessary therefore to give a reasonable chance of perfect matching between recipient and donor. The difficulty of achieving this limits the value of the scheme.

An alternative approach to the problem of rejection is the use of immunosuppressives. These can be categorised broadly as those with a specific and those with a non-specific mode of action. Specific immunosuppressives do not interfere with antibody response to infection and do not produce overall depression of cell mediated immunity. Non-specific immunosuppressives on the other hand produce overall depression of both humoral and cell mediated immunity. If immunosuppression is profound overwhelming infection is liable to occur (Evans, 1969), and interference with cell mediated immunity increases the likelihood of neoplasia arising spontaneously in the recipient. This occurrence has been reported on numerous occasions (Doak, Montgomerie, North and Smith, 1968; Woodruff, 1968), and has been reviewed by Penn (1971) and by Smellie (1971). Penn (1971) found a 6.7 per cent incidence of malignancy in immunosuppressed renal allograft recipients. This compared with an

incidence of malignancy in the general population of similar age of 58 per 100,000. The frequent occurrence of reticulum cell sarcomas in renal allograft recipients was also noted. In a review by Schwartz and André-Schwartz (1968) evidence is presented suggesting that immunosuppression may encourage oncogenic viruses to induce lymphoproliferative malignancy.

In spite of these disadvantages non-specific immunosuppression has an established place clinically in the management of transplant recipients. The purine analogue azathioprine (Imuran) was shown by Calne and Murray in 1961 to produce prolongation of survival of canine renal allografts. It has been used clinically since 1962 and remains the most widely used immunosuppressive drug.

X-irradiation also has a non-specific immunosuppressive action. Murray, Merrill, Dammin, Dealy, Walter, Brooke and Wilson (1960) reported the results of a clinical trial of immunosuppression by total body irradiation and noted a high incidence of bone marrow depression and overwhelming infection. Immunosuppression by this means is no longer advocated. Local irradiation to the graft was evaluated by Hume, Magee,

Kauffman, Rittenbury and Prout (1963) and has been used with success in some centres (Starzl, 1964; Calne, 1967).

Corticosteroids have been employed for a number of years as an adjunct to other immunosuppressive drug therapy, but they have not proven their value as primary immunosuppressive agents.

The capacity of heterologous antilymphocyte serum (ALS) to delay rejection of allografts was first demonstrated by Woodruff and Anderson in 1964, and represents an important advance in immunosuppression. Although ALS is essentially non-specific in its mode of action it has the advantage of acting primarily on cells responsible for mounting cellular immune reactions.

The complications attended by the use of non-specific immunosuppressive agents has focused attention on the capacity of specific phenomena including immunological enhancement and tolerance to induce suppression of allograft rejection. By such means it might be possible to prevent rejection without interfering with the overall immunocompetence of the graft recipient.

The object of experiments presented in this thesis is the demonstration and investigation of prolongation of

cardiac allograft survival in rats which follows pre-treatment of recipients with donor strain cells. An attempt has been made to establish the mechanism of the effect.

The presence of a number of inbred strains make the rat an ideal experimental animal when strict standard-isation of experiments is required, and microvascular techniques have made heterotopic heart transplantation possible in this species. A highly satisfactory and reproducible experimental model has resulted.

Calne (1971) has drawn attention to the spectrum of susceptibility to rejection which exists between skin, heart, kidney and liver allografts within a species, and noted that liver transplants are rejected least aggressively. This serves as a reminder that experimental results obtained using allografts of one tissue may not be valid for another. As well as this species differences are of obvious importance, and it would be unwise to apply experimental results using rats equally to man or to other species since quantitative or qualitative differences of immunological response may exist between them. It is of interest that unlike the rat human recipients of renal allografts who have

detectable cytotoxic antibody before transplantation reject their grafts in a hyperacute fashion (Kissmeyer-Nielsen, Olsen, Petersen and Fjeldborg, 1966; Williams, Hume, Hudson, Morris, Kano and Milgrom, 1967; Terasaki, Thasher and Hauber, 1968; Russell, 1971).

In spite of this warning note it is possible that specific immunosuppression in some degree may be applicable to clinical transplantation. It is hoped that the experiments in this thesis at least in a small way will help to promote further interest and research into a phenomenon of such possible potential importance.

C H A P T E R I

A review of the literature

THE DEVELOPMENT OF THE CONCEPT OF IMMUNOLOGICAL ENHANCEMENT

The phenomenon of enhancement was first noted by Flexner and Jobling (1907) who found that growth of a rat sarcoma was favoured if the host was pre-treated with an emulsion of the tumour cells which had been heated at 56°C for half an hour. The first systematic study was by Casey (1932, 1934a, 1934b, 1941) and Casey, Meyers and Drysdale (1948). They found that Brown-Pearce carcinoma of rabbits grew and metastasised more rapidly and widely in recipients pre-treated with killed homologous tumour tissue. They also demonstrated the specificity of the effect using transplantable tumours in mice.

This was followed by reports which showed that the effect on mouse tumours could be produced equally well using donor strain spleen, kidney, and liver cells as distinct from tumour tissue to pre-treat the tumour host (Kaliss and Snell, 1951; Snell, 1952; Day, Kaliss, Aronson, Bryant, Friendly, Gabrielson and Smith, 1954). These findings indicated that the effect was not induced by a growth promoting agent contained in the tumour extract, and also that tumour-specific antigens are not relevant to enhancing phenomena.

Using rats and mice Kaliss and Molomut (1952), Kaliss, Molomut, Harriss and Gault (1953) and Kaliss (1955) demonstrated that enhanced tumour growth could be passively transferred by injecting serum from animals of the same strain which had been subjected to enhancing treatment. The globulin fraction of serum was shown by Kaliss and Kandutsch (1956) to possess this potential.

In recent years numerous studies of enhancement have been carried out using normal tissue allografts instead of tumours and have been reviewed by Batchelor (1963), Kaliss (1969) and Billingham and Silvers (1971). Billingham and Sparrow (1955) prolonged survival of rabbit skin grafts by prior treatment of the recipient with dissociated epidermal cells given intravenously. Since that time numerous workers have demonstrated enhancement of skin allografts (Shapiro, Martinez, Smith and Good, 1961; Guttman and Aust, 1961; Nelson, 1962; Möller, 1964b).

Following the introduction of microvascular surgical techniques in 1960 it became feasible to transplant organs in small laboratory animals by vascular anastomosis, and within a few years Stuart, Saitoh, Fitch and Spargo (1968) reported prolongation of survival

of rat renal allografts using pre-treatment schedules of antiserum alone, donor strain spleen cells alone, and a combination of these agents in three groups of recipients. The combined treatment was found to induce marked prolongation or indefinite survival of the grafts.

Since this important study others have demonstrated enhancement of organ allografts. Marquet, Heystek and Tinbergen (1971) produced prolonged survival of rat cardiac and renal allografts by pre-treatment of recipients with donor strain blood. Jenkins and Woodruff (1971) using cardiac allografts confirmed this finding with a different strain combination and demonstrated that a similar effect occurred after injection of donor strain thoracic duct lymphocytes. Lucas, Markley and Travis (1970) and Ockner, Guttman and Lindquist (1970a) also reported prolongation of survival of rat renal allografts following pre-treatment of recipients with donor strain spleen and bone marrow cells, and Halasz, Orloff and Hirose (1964) demonstrated delayed rejection of dog renal allografts after pre-treatment of recipients with donor blood.

POSSIBLE MECHANISMS OF IMMUNOLOGICAL ENHANCEMENT

Since the immunological nature of enhancement was demonstrated by Kaliss and Snell (1951) numerous theories of the mechanism of the phenomenon have been advanced and have been reviewed by Kaliss (1962), Batchelor (1963), Hutchin (1968), Kaliss (1969), Amos, Cohen and Klein (1970), Snell (1970), Russell (1971), and Voisin (1971). Following the separation of the immune response into cellular and humoral components, Billingham, Brent and Medawar (1956) suggested that humoral antibodies might in some way block the destructive effect of a cell mediated response on an allograft. The "blocking theory" of enhancement arose from this concept, and Billingham and his associates suggested that afferent, central, or efferent block of the cellular immune response might occur. Afferent blockage implies a block between the original antigen depot and the immune centres. Central blockage is a block "which affects the actual machinery of antibody formation or of some equivalent immunological process". Efferent blockage is a block "which in some manner prevents the effectors of the immune reaction from

exercising their action".

Snell, Winn, Stimpfling and Parker (1960) employed the Winn Assay of cell mediated immunity (Winn, 1960) to demonstrate that passively transferred antiserum reduced the cellular immune response to the antigen against which the antiserum was raised. It was deduced that the blockage was therefore afferent or central. This work was extended by Takasugi and Hildemann (1968) who showed that passively transferred antibody reduced the lymphocytosis induced by a graft, and also reduced the immunity shown by circulating lymphocytes. Snell's deduction that the block was either afferent or central was therefore confirmed.

In order to differentiate between an afferent or a central block Takasugi and Hildemann (1968) removed allografts six days after transplantation. This did not prevent the lymphocytosis from developing until antibody was added. It was deduced that the block was probably central. However there is also good evidence that a block of the efferent path may occur too. Möller (1963, 1964a) injected mice in the flanks with allogeneic tumour cells. On one side the cells were coated with allo-antibody. Subsequent growth occurred on the treated

side, but not on the untreated side. Central and afferent blocks are clearly excluded in this system.

Most workers now agree that while there is little evidence that enhancement is produced by an afferent block, both central and efferent blocks of the immune response may occur, possibly depending upon the system used.

An alternative approach is suggested by Guttman, Lindquist and Ockner (1969) who have demonstrated the importance of donor passenger leucocytes within allografts. They suggest that such cells act as the most powerful immunogens within a graft, and that the action of enhancing antibody may be to eliminate these cells.

The fundamental nature of enhancement has been suggested by Hellström and Hellström (1970) and Hellström, Hellström and Allison (1971) in experiments which have linked enhancement to neonatally induced tolerance. They showed that lymph node cells from CBA strain mice made tolerant to A strain as neonates were cytotoxic to A strain cultivated fibroblasts in the presence of control serum. The addition of serum from tolerant CBA mice nullified this effect which was also shown to be specific. The inference drawn was that the tolerance

of the CBA mice was due solely to serum factors. The borderline between tolerance and enhancement therefore seems to be indistinct.

THE DEVELOPMENT OF HETEROTOPIC HEART TRANSPLANTATION IN THE RAT

Anastomosis of small vessels has presented serious technical problems for many years. This is reflected by the numerous methods of anastomosis which have been advocated, some of which involve vessel suturing and some the use of splints, rings, staples and tissue adhesives (Mozes, Man, Agmon and Adar, 1963; Rohman, Goetz and Dee, 1960; Androsov, 1956; Hafner, Fogarty and Cranley, 1963). In general non-suture methods have proved unsatisfactory for small vessel work and refinements of technique and instruments have shown that the best results are obtained by suturing.

The obvious value to immunological research of organ transplantation in small laboratory animals has stimulated the testing of numerous different operative methods.

Heart transplantation by implantation of the graft was used by Judd and Trentin (1969) in mice. This involves implantation of foetal hearts into the subcutaneous tissue of the pinna of the ear of adult recipients. A similar technique was evaluated by the

author (unpublished data) using rats. The grafts pulsate visibly and transmit good E.C.G. recordings (Plate 1). The advantage of the method lies in its simplicity, though it is not generally used since the mode of vascularisation of the grafts is very different from that in clinical heart transplants.

Transplantation of the rat heart into the neck was described by Heron (1971). Vascular anastomosis was achieved using extravascular Teflon prostheses making it unnecessary to employ microvascular suturing techniques. A 10 to 15 per cent incidence of thrombosis was noted.

Sophisticated microvascular techniques were first employed for transplantation of the rat heart by Abbott, Lindsey and Creech (1964). Transplantation was performed by anastomosis of the donor thoracic aorta to recipient abdominal aorta, and donor pulmonary artery to recipient inferior vena cava, both in an end-to-end fashion. Interruption of blood supply to the recipient's caudal region led to a high incidence of post-operative paraplegia. Modification to end-to-side anastomosis avoiding transection of the recipient's great vessels was shown by Bui-Mong-Hung and Vigano (1966), and by Ono and

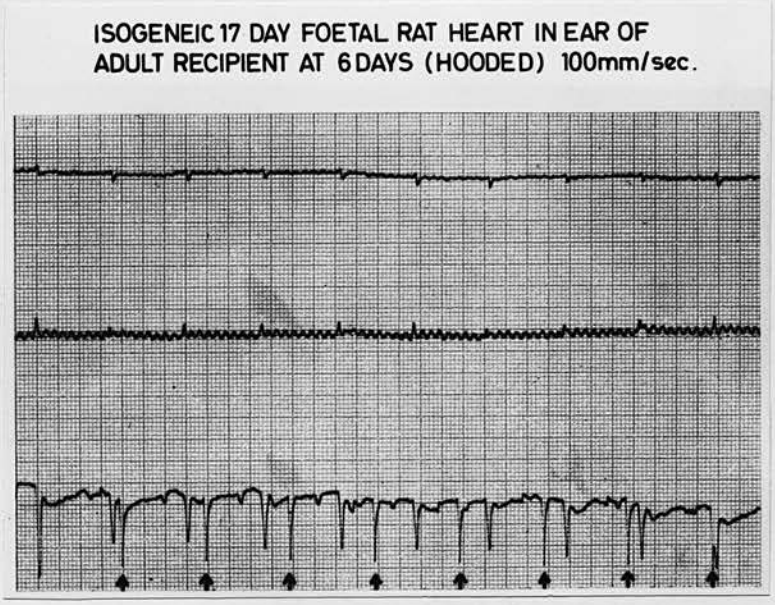


Plate 1: ECG recording of foetal rat heart implanted in ear of adult recipient (isogeneic). Upper and lower limb leads placed on cheeks and neck of recipient. Chest lead transfixed the ear containing the transplant. Arrows mark complexes produced by the transplant.

Lindsey (1969) to produce markedly improved results. This technique is now used in several centres and has proved satisfactory (Jenkins and Woodruff, 1971; Marquet, Heystek and Tinbergen, 1971). It was shown by the author (unpublished data) to be technically feasible in the mouse also, though as the diameter of the aorta is about 0.25 mm. a special degree of surgical precision is necessary (Plate 2).

Transplantation of the rat heart by this method has been used throughout the present study.

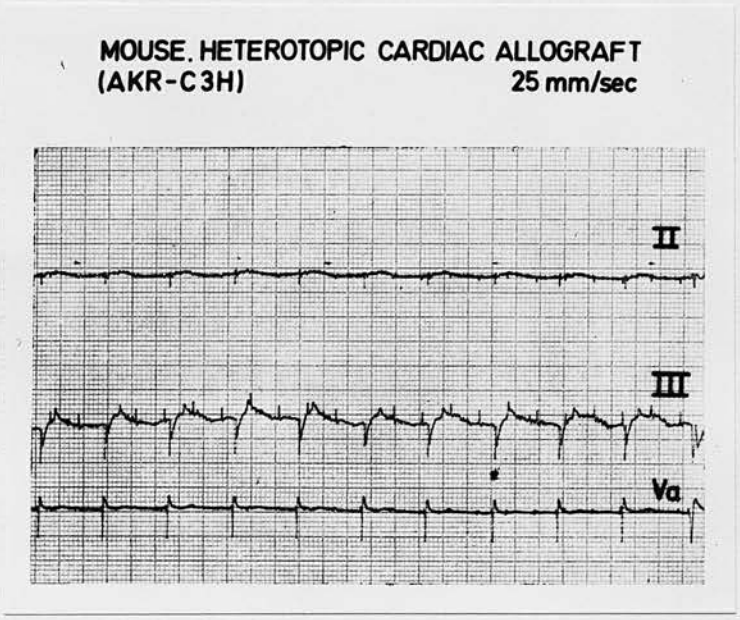


Plate 2: ECG recording of mouse heterotopic cardiac allograft established by vascular anastomosis into abdomen of recipient. Chest lead was placed over the transplant. The lowest tracing shows complexes produced by the transplant.

C H A P T E R II

Materials and Methods

1. RATS

a) A2/1 strain (Plate 3)

This strain was produced from a substrain of Wistar rats known as A_2 by Michie and Anderson (1966) at the University of Edinburgh. In spite of a record of 72 generations of brother-sister matings about 50% of skin grafts exchanged between A_2 rats were rejected. This was found to be due to a powerful genetic stabilising effect known as heterosis which resulted in almost every surviving rat being heterozygous, and further studies showed that the selective elimination of the homozygous state occurred at fertilisation.

A new breeding policy resulted in the identification of a few rats which accepted skin grafts from both parents. These were then brother-sister mated and free acceptance of grafts between the progeny showed that an isogeneic substrain had been produced and which has been known subsequently as A2/1.

Histocompatibility typing of A2/1 rats has been performed by Palm (1970) who has shown that they carry the AgB_2 histocompatibility antigen. Anderson, James and Woodruff (1967) have shown that this strain rejects

Plate 3: The albino A2/1 rat.

split-thickness skin grafts from Hooded rats in 8.6 days.

In experiments reported in this thesis A2/1 strain male animals (180-250 G) have acted as donors of cardiac and skin allografts.

b) Hooded strain (Plate 4)

This strain has been maintained in this laboratory for over 13 years by brother-sister matings. The rats are isogeneic and rejection of skin grafts within the strain does not occur. The strain is known to carry the AgB_5 histocompatibility antigen, and Anderson, James and Woodruff (1967) have shown that they reject A2/1 split-thickness skin grafts in 8.1 days.

Male Hooded rats (180-250 G) have acted as recipients of heart and skin allografts in these experiments. They have also been used in some experiments to raise alloantiserum.

c) F_1 hybrid rats

F_1 hybrid rats were bred by mating a Hooded male and A2/1 female for one generation in the Department of Surgery, University of Edinburgh.

Plate 4: The Hooded rat.

d) Sprague-Dawley rats

In one experiment male rats of this strain (AgB_6) have been employed to provide third party blood.

e) Lewis strain

Male rats of this strain also have been used to provide third party blood, and were generously provided by Professor K. Porter, St. Mary's Hospital Medical School, London. The histocompatibility status of this strain is AgB_1.

2. HETEROTOPIC HEART TRANSPLANTATION

a) Anaesthesia

Donor animals

Anaesthesia was induced in an ether chamber for all donor animals. Following shaving of the abdomen and chest the animals were pinned to an operating board and anaesthesia was maintained by means of a nose-cone containing ether. This type of anaesthetic was also used for other procedures such as thoracic duct cannulation and skin grafting.

Recipient animals

0.1-0.15 ml. of veterinary nembutal, depending on the weight of the rat, given intraperitoneally was used for all heart recipients. This anaesthetic agent was found to be reliable, and with experience a dose could be estimated which would allow the animal to wake at the time that the operation ended. On occasion supplementary ether was required. It was found that the use of nembutal as the main anaesthetic agent instead of ether resulted in a lowering of respiratory problems during and after transplantation.

b) Instruments and equipment used in transplantation

1. Operating microscope

In order to obtain technically perfect vascular anastomoses a Zeiss binocular operating microscope was used for all cardiac transplants (Plate 5). The instrument is a diploscope which enables an assistant to help when necessary. However except in heart re-transplant operations assistance was not used. The operating microscope has a wide range of magnification available, from x6 to x40, but x10 magnification was found to be optimum. The illumination does not produce appreciable heating of the operative field and does not therefore cause drying of the tissues.

2. Operating instruments (Plate 6)

No instruments specially made for microvascular work were used. Ophthalmic instruments were found to be satisfactory, and these included iris scissors for opening the recipient vessels, Castroviejo pattern needle-holding forceps, and very fine tissue forceps. Castroviejo corneal scissors were found ideal for transecting the donor aorta and pulmonary artery at the level of the transverse sinus.

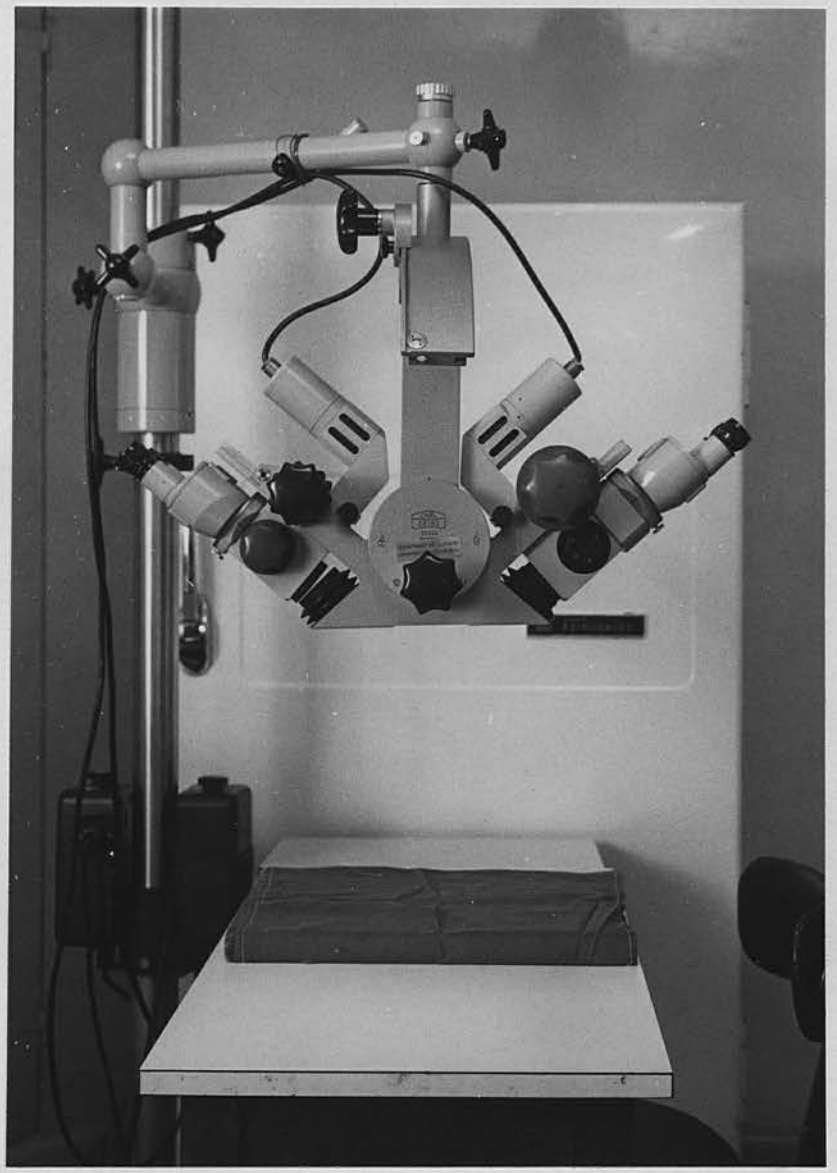


Plate 5: Zeiss binocular operating microscope.

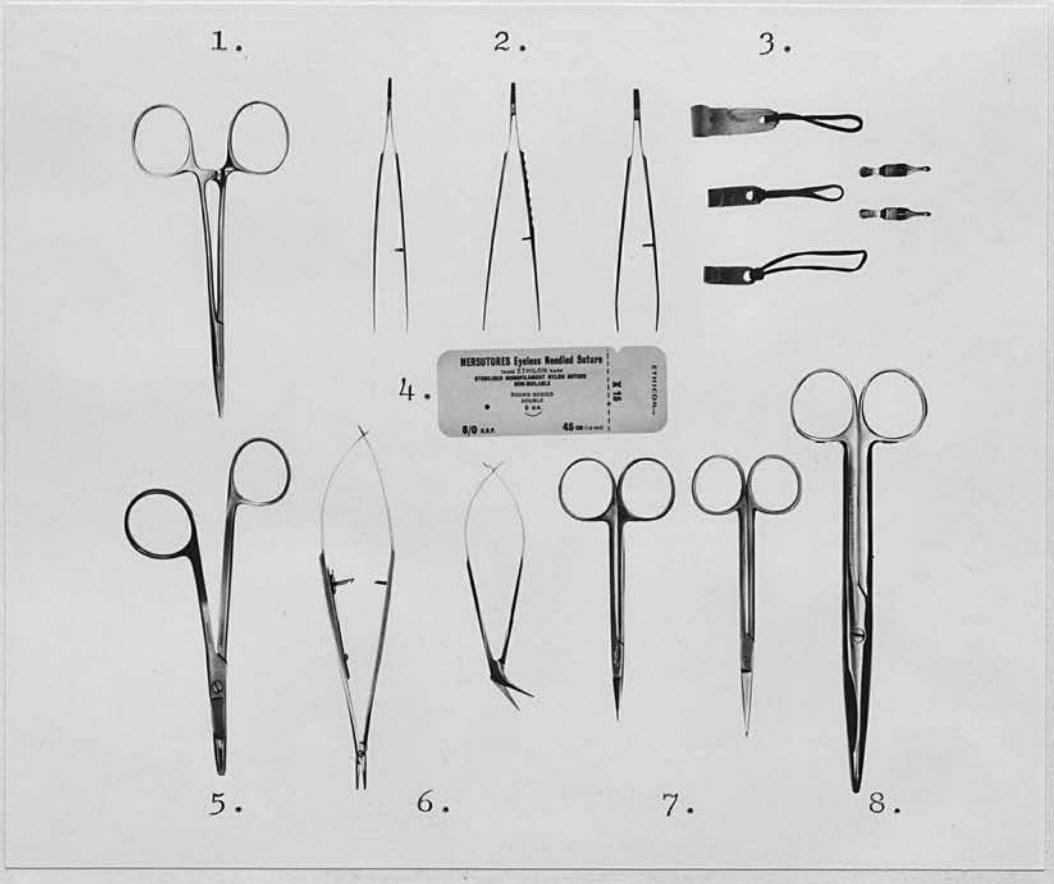


Plate 6: Instruments and suture material used in heterotopic heart transplantation.

1. Artery forceps
2. 3 fine dissecting forceps (plain, toothed, curved)
3. Retractors and vascular clamps
4. Suture material
5. Needle-holder-scissors
6. Castroviejo needle-holder and corneal scissors
7. Two pairs fine iris scissors
8. Mayo scissors

Small bulldog vascular clamps served to occlude the recipient vessels during transplantation. Small pellets of Kleenex tissues were found to absorb extravasated blood more satisfactorily than cotton wool pellets.

3. Suture materials

8/0 Ethilon (Ethicon) monofilament nylon sutures mounted on a curved 8 mm. round bodied needle were used for all anastomoses (Plate 7). This material was found to be more suitable than silk which tended to saw through fine vessels. Its slight springiness made picking up with needle-holding forceps during knot tying easy as unlike silk it does not adhere to surrounding tissues. With this fine grade of nylon no tendency for knots to slip was noted.

5/0 braided silk was used for tying small communicating vessels and for ligating donor cavae and pulmonary veins.

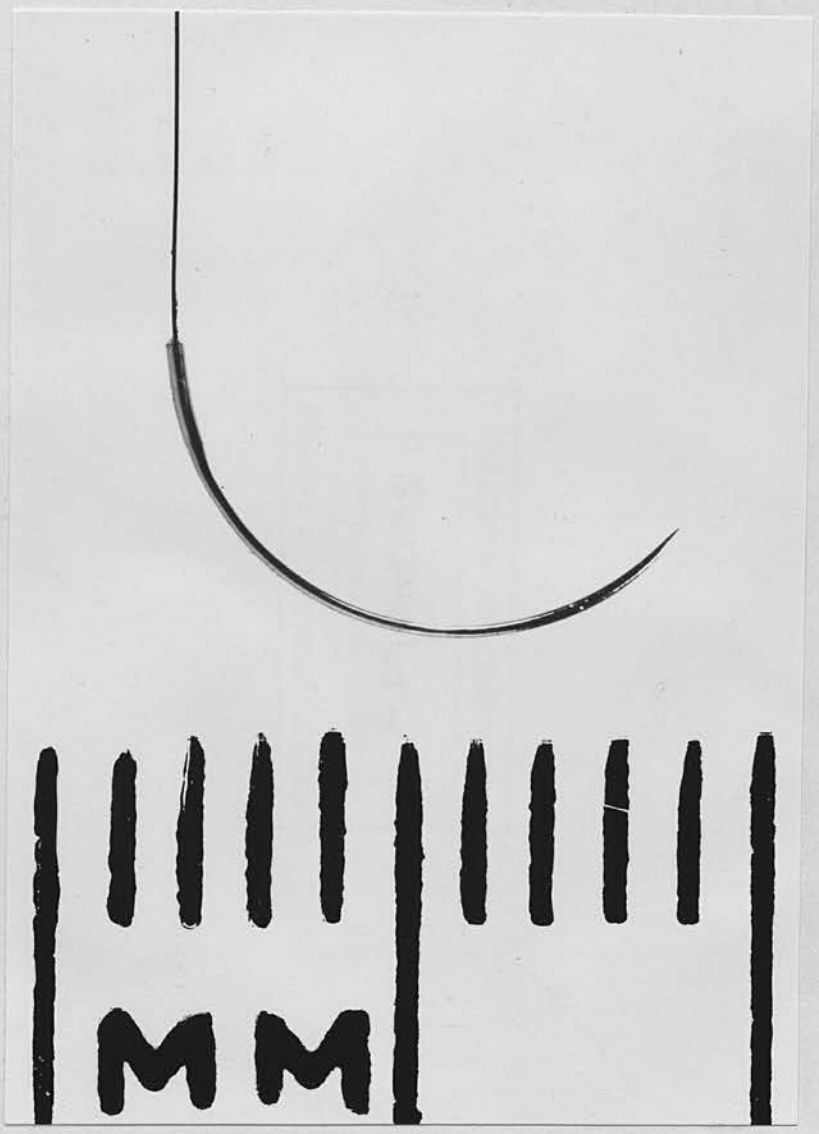


Plate 7: Monofilament nylon suture mounted on a curved round bodied 8 mm. needle. (approx. x10 magnification)

c) Operative technique

The technique employed in these studies was similar to that described by Bui-Mong-Hung and Vigano (1966) and Ono and Lindsey (1969), in which end-to-side anastomoses are made between donor ascending aorta and recipient abdominal aorta, and between donor pulmonary artery and recipient inferior vena cava. The donor cavae and pulmonary veins are ligated allowing a simple coronary perfusion of the graft. Such grafts are fully pulsatile on restoration of circulation.

(i) Donor animal

Heparinisation. The mesentery is exposed through a midline abdominal incision and 300 units of heparin are injected into a mesenteric vein.

Exposure. The rib cage is divided along its lateral aspects and the anterior fibres of the diaphragm are detached from the anterior chest wall. The resulting flap of chest wall is hinged in a cephalad direction to expose the heart and is retained by means of a needle which transfixes the xiphisternum and passes into the operating board (Plate 8).

Removal of the heart. The pericardium is

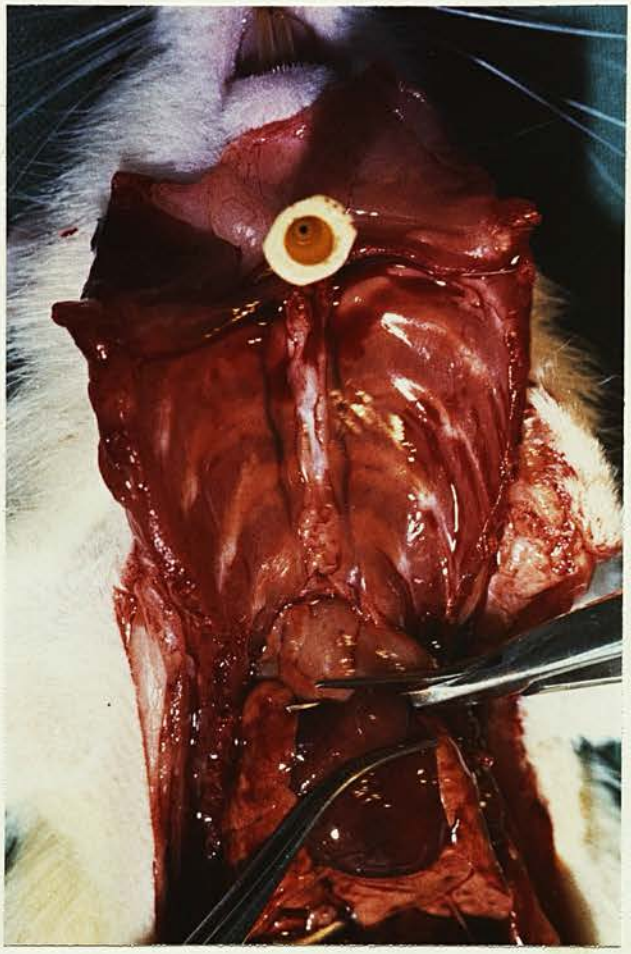


Plate 8: Removal of heart from donor rat. The anterior chest wall is hinged in a cephalad direction. A scissor blade is passed through the transverse sinus and the aorta and pulmonary artery are transected together.

opened and the inferior vena cava is ligated with 5/0 silk and is divided. A blade of a pair of corneal scissors is passed through the transverse sinus of the heart, and the ascending aorta and pulmonary artery are transected together. A silk ligature is passed round the pulmonary veins and inferior cavae, and these vessels are divided en masse distal to the ligature.

Perfusion and immersion of the isolated heart. Perfusion of the aortic stump with 2 ml. of 0.9% saline at $4^{o}C$ is carried out through a Pasteur pipette. This causes myocardial contractions to cease. The heart is placed in cold saline for an average of 8 minutes before transplantation.

(ii) Recipient animal

Position and incision. The animal is placed supine on the operating board and the hind limbs are secured to the board with rubber leg bands. The fore limbs are left free to avoid impeding respiratory movement. A midline xipho-pubic incision is used and retractors are inserted to give exposure.

Exposure of aorta and vena cava. The gut is packed in damp swabs and the mesocolon is divided giving

access to the abdominal aorta and vena cava. Peritoneum and fat are wiped from these vessels with pellets of rolled Kleenex tissues, and the splanchnic nerves lying on the aorta are stripped from the vessel and divided (Plate 9). A point midway between the origin of the renal vessels and the aortic bifurcation is selected for anastomosis of the donor heart, and aortic branches and caval tributaries are ligated in that segment. Two small vascular clamps are used to isolate the segment.

Anastomosis. The aorta and vena cava are opened through small vertical incisions. The donor and recipient aortae are approximated with two stay sutures, and the anastomosis is completed with a continuous suture of 8/0 Ethilon (Plates 10, 11, 12). On preparing to insert the last stitch of both anastomoses it is necessary to leave the previous stitch loose so that the vascular lumen can be identified otherwise the opposite wall of the vessel may be picked up with the needle with obliteration of the vascular lumen. In each case the vessel lumen is filled with saline prior to closure. If this step is omitted air embolism of the donor myocardium destroys a proportion of grafts, and fatal air embolism has been noted from the venous anastomosis.

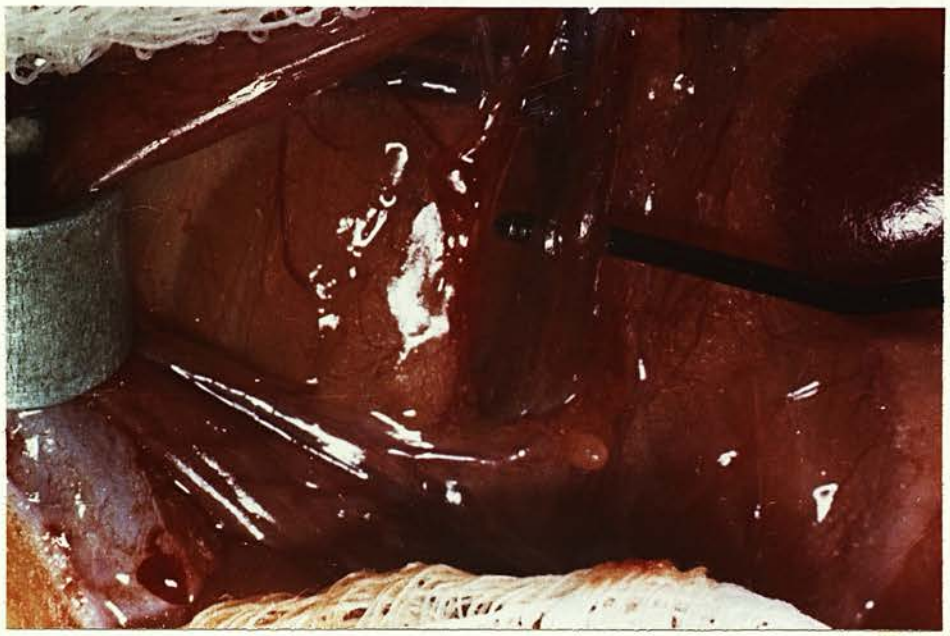


Plate 9: Exposure of the recipient's inferior vena cava and aorta. The colon is retracted to give exposure. The lower pole of the left kidney is visible.

Plate 10: End to side aortic anastomosis. Completion of the first side between upper and lower stay sutures.

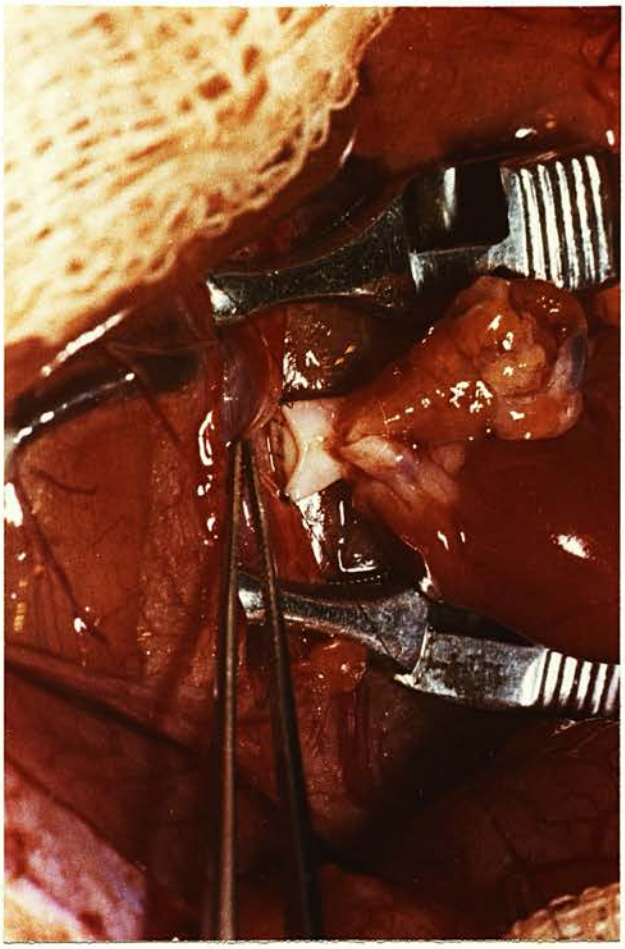


Plate 11: End to side aortic anastomosis prior to suturing of the second side. The heart is reflected to the left and the lumen of recipient and donor aortae is exposed.

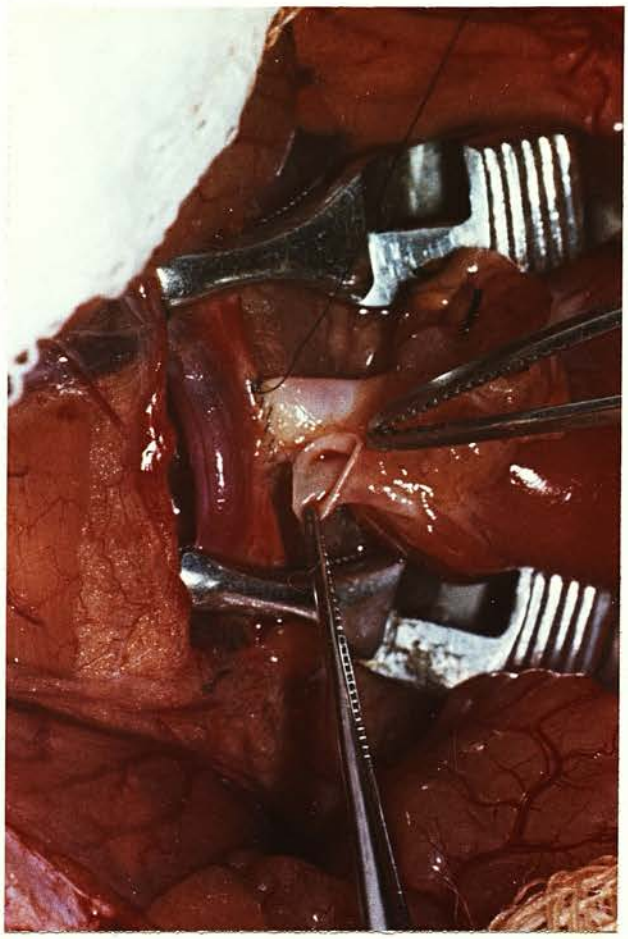


Plate 12: Completion of aortic anastomosis. The pulmonary artery is prepared for anastomosis to the recipient vena cava.

The left side of the venous anastomosis is fashioned from within the lumen of the vessels since the aortic anastomosis prevents normal access. A similar continuous suture is again employed (Plates 13, 14, 15).

Re-establishment of graft circulation (Plates 16, 17, 18). The distal clamp is removed first, and light pressure with pledgets controls oozing of blood. Coordinated ventricular contractions return spontaneously a few moments after removal of the clamps, but in some instances it is preceeded by a brief period of ventricular fibrillation (Plate 19). The average period of anoxia of grafts is 25 minutes.

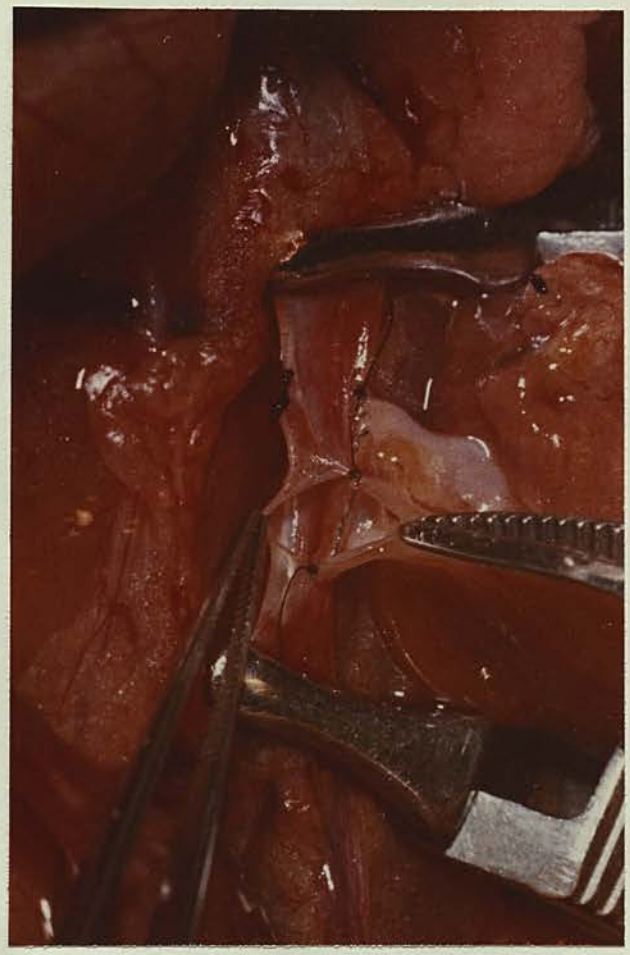


Plate 13: Anastomosis of pulmonary artery to inferior vena cava. Completion of the first side which is performed from within the vascular lumen.

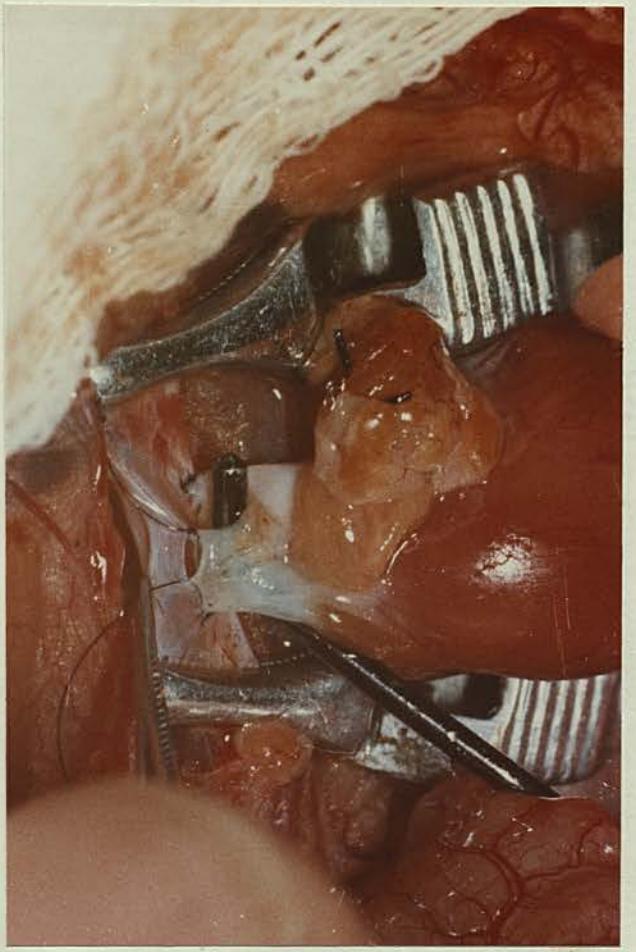


Plate 14: Anastomosis of pulmonary artery to inferior vena cava. Second side nearing completion. A probe has been passed between the aortic and venous anastomoses.

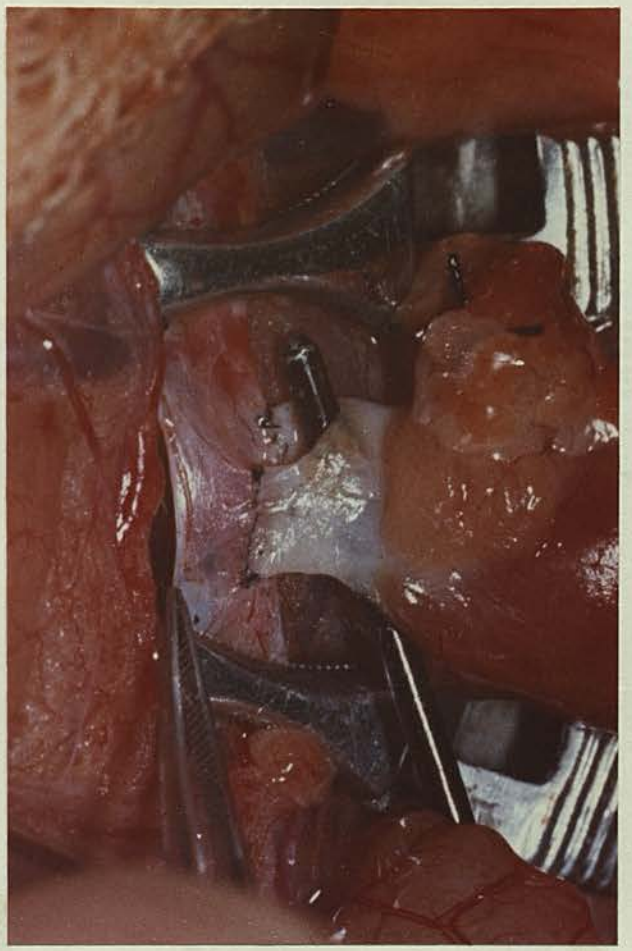


Plate 15: Both anastomoses complete.

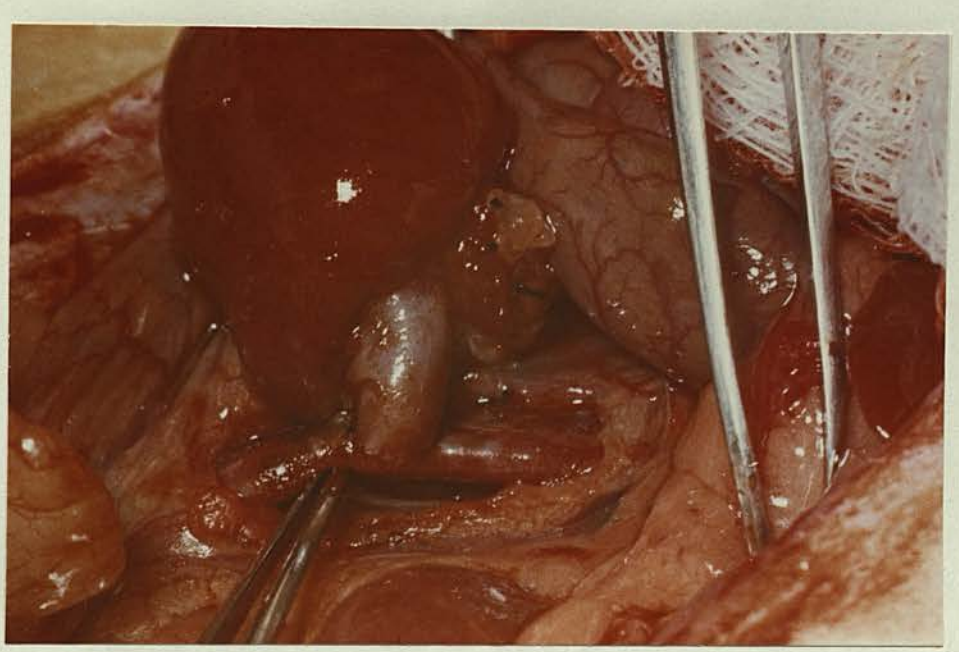


Plate 16: Aortic anastomosis following removal of vascular clamps.

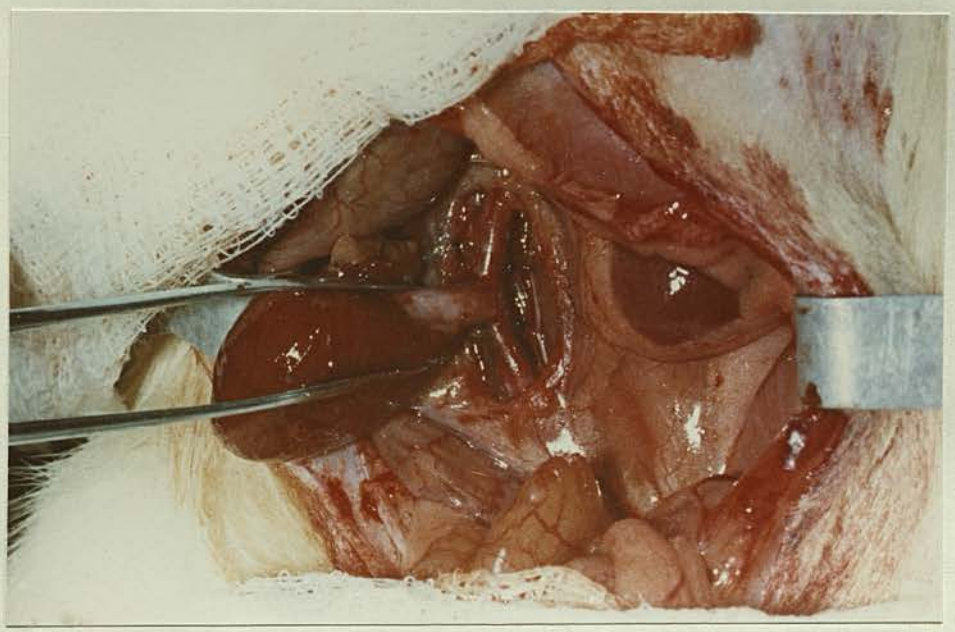


Plate 17: Transplant in relation to viscera. Colon to left, stomach above and to right, kidney to right, seminal vesicles below.

Plate 18: The transplanted heart is allowed to lie free among the coils of small bowel.

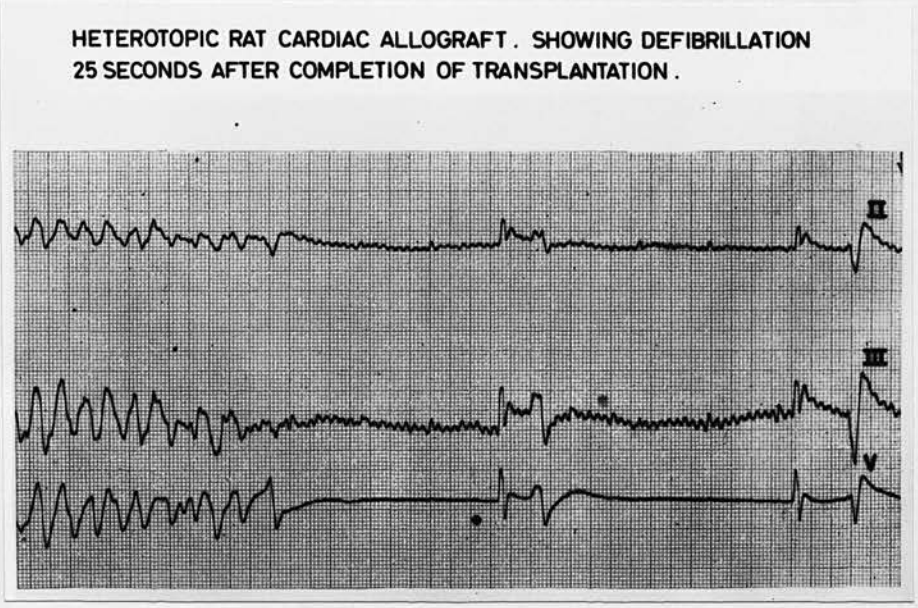


Plate 19: ECG recording of heterotopic heart transplant shortly after removal of the vascular clamps. Spontaneous defibrillation is shown occurring 25 seconds after circulatory return.

3. ASSESSMENT OF GRAFT ACTIVITY

Grafts were considered to have been rejected when no pulsatile activity was present, and an E.C.G. recording was flat. Rejection of hearts was subsequently confirmed histologically.

a) Palpation. This was carried out daily, other than with hearts active for in excess of 4 weeks. These were examined thrice weekly. On occasion the recipient had to be anaesthetised especially when the amplitude of pulsation had diminished as before rejection. It was found that the force of the blood entering an inactive graft could give an erroneous impression of contractibility, and that this factor could be eliminated by pressure occlusion of the recipient's aorta proximal to the graft during palpation.

b) E.C.G. A direct writing ink jet electrocardiogram (Mingograf, Sierex) was used throughout the study (Plate 20). Animals subjected to this examination were anaesthetised briefly with ether. As shown by Abbott, DeWitt and Creech (1965) and Bui-Mong-Hung and

Plate 20: The Mingograf (Sierex) electrocardiogram in use.

Vigano (1966) positioning of the upper limb leads in the subcostal region or near the iliac crests, and the exploring electrode over the transplant served to suppress or eliminate interference from the animal's own heart (Plate 21). By adjusting the position of the exploring electrode activity from the animal's own heart and that from one or more transplants could be recorded on one tracing (Plates 22 and 23). A newly transplanted heart adopts a regular rhythm independent of the animal's own heart, but often approximating to it in rate. Preceeding this there is frequently a variable period of bradycardia associated with defective intraventricular conduction. This may be caused by the trauma and anoxia of transplantation. Abbott, Creech and DeWitt (1965) showed that isografts produce a relatively normal E.C.G. tracing indefinitely. During rejection of allografts the E.C.G. tracing shows progressive loss of amplitude of QRS complexes, defective intraventricular conduction, marked bradycardia and finally when rejection is complete, a flat tracing.

c) Histology. At the time of rejection control allografts showed a severe allograft reaction with

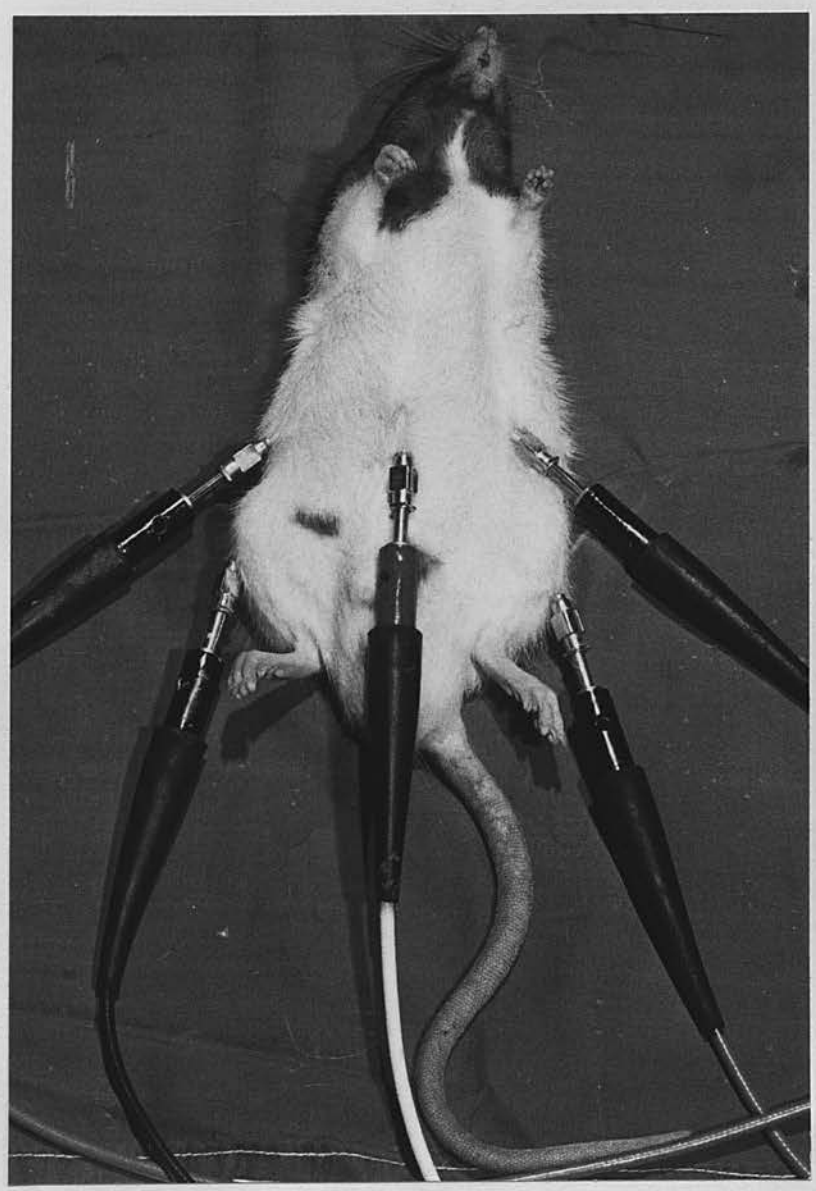


Plate 21: Positioning of ECG leads for recording of a cardiac transplant. The upper limb leads are placed in the subcostal area, and the exploring electrode over the transplant.

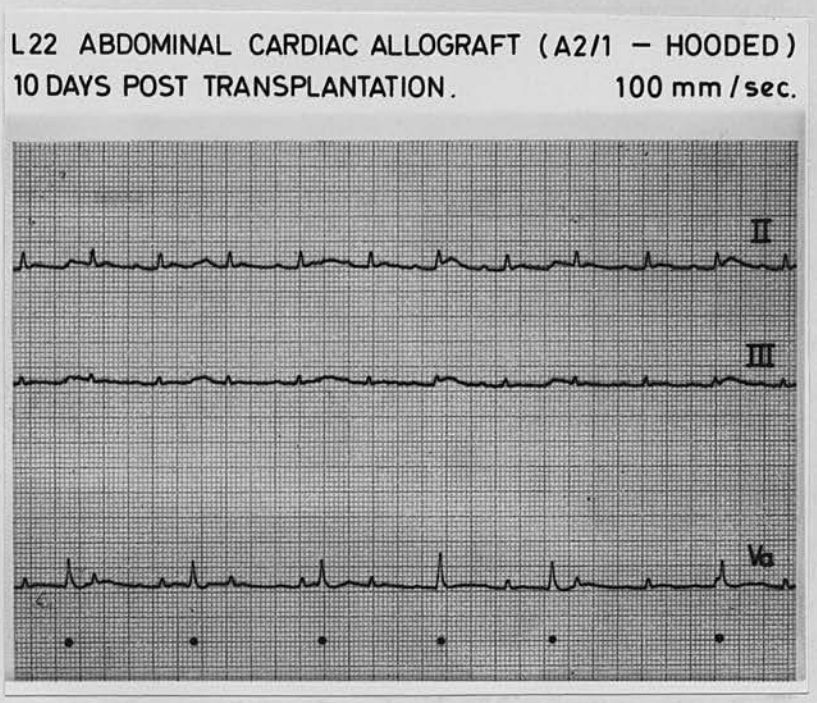


Plate 22: ECG recording (100 mms/sec) showing complexes produced by a single heterotopic cardiac allograft in the lower tracing (marked with .).

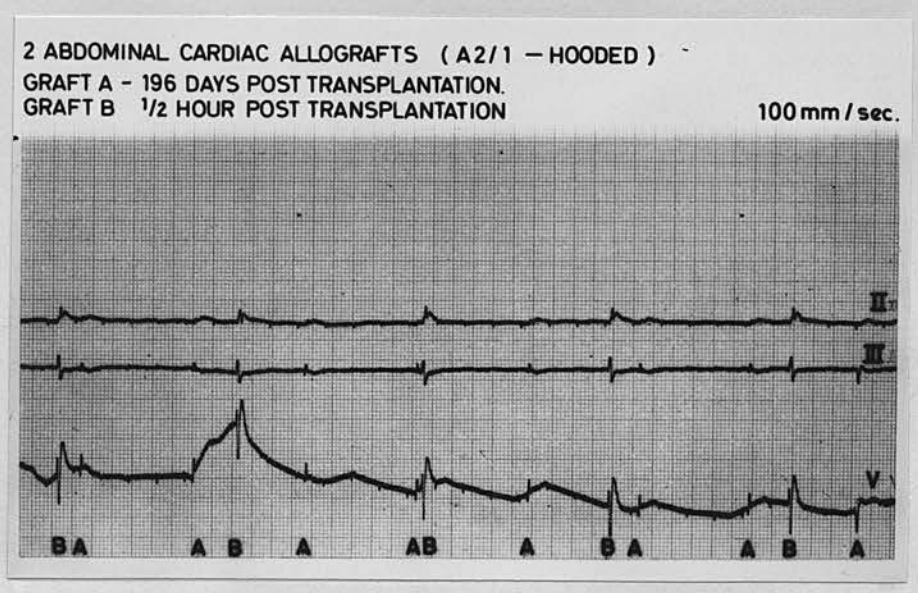


Plate 23: ECG recording (100 mms/sec) showing complexes produced by 2 heterotopic cardiac allografts (A and B) in the lower tracing.

Graft A - 196 days post transplantation
Graft B - $\frac{1}{2}$ hour post transplantation

destruction of much of the muscle component as observed by Abbott, Creech and DeWitt (1964), Abbott, DeWitt and Creech (1965) and van Bekkum, Heystek and Marquet (1969) (Plate 24). Such hearts had marked oedema of the myocardium associated with a diffuse cellular infiltrate. Scattered areas of haemorrhage also occurred. The cellular infiltrate consisted of lymphocytes and plasma cells but foci of polymorphonuclear cells were also present around areas of muscle necrosis. There was little intimal proliferation of the coronary vessels of hearts which were rejected acutely.

In contrast those hearts which were subjected to chronic rejection showed myocardial destruction with extensive fibrous replacement. There was marked coronary arteritis with intimal proliferation and frequently obliteration of the vascular lumen (Plate 25).

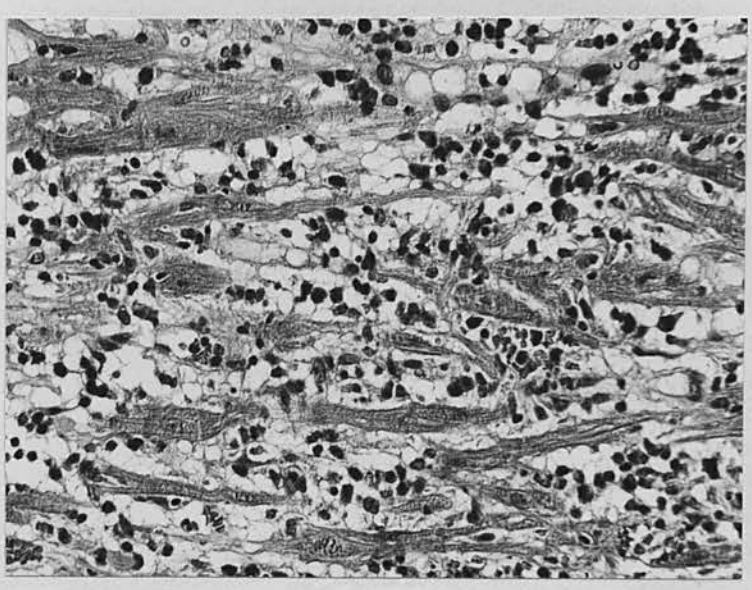


Plate 24: Myocardium of cardiac allograft 14 days after transplantation showing marked destruction of the muscle component with cellular infiltration.

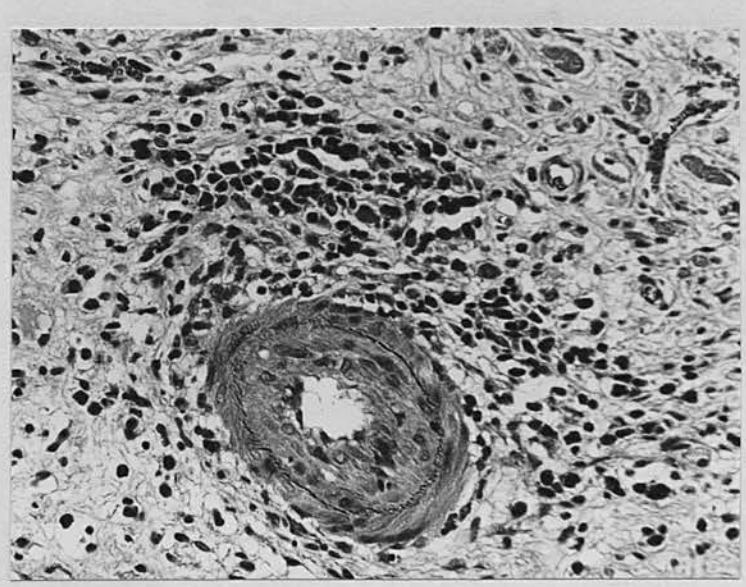


Plate 25: Myocardium and coronary vessel of cardiac allograft 162 days after transplantation into recipient pre-treated with donor strain blood. There is obliteration of the muscle component and marked coronary arteritis.

4. TECHNIQUE OF SPLIT SKIN GRAFTING IN THE RAT

The technique used in these studies is similar to that described by Woodruff and Simpson (1955).

Preparation of the Recipient

Open ether anaesthesia is employed and the animal is laid on its side on an operating board. The hair is shaved from the side of the thorax and the skin is cleaned with spirit. Observing strict aseptic precautions and with the skin placed under tension by an assistant a square of skin measuring 2 cm. x 2 cm. is outlined using a scalpel as described by Taylor and Lehrfield (1953). A corner of the square of skin is pushed up with a sharp Gillies skin hook and is placed on tension. The panniculus carnosus is pushed away with a pledget until the whole square of skin has been removed. By this means little bleeding occurs, and the fine vessels coursing across the panniculus are not disturbed.

Technique of obtaining split skin grafts

With donor animal anaesthetised and prepared in the same way as the recipient, the skin at the selected site is painted with dermatome adhesive after cleansing with

ether. A McIndoe pattern dermatome was employed in these studies using a blade-drum clearance of 0.008 inch as recommended by Woodruff and Simpson (1955). Once cut the graft is laid on its epithelial aspect on a square of Sofra Tulle vaseline gauze and is smoothed out and is cut to the desired size.

Technique of applying and securing split skin grafts

The graft is laid into the prepared skin defect in the recipient animal and is secured by a number of fine silk sutures. The graft is protected by the application of a plaster of Paris bandage which is removed 7 days later.

Evaluation of graft viability

Grafts were examined daily after removal of the dressings on Day 7. Rejection was assessed macroscopically and was considered to have taken place at the time when destruction of the epithelium appeared complete.

5. COLLECTION AND PREPARATION OF DONOR STRAIN MATERIALS

a) Blood, plasma and serum

Donor strain animals were exsanguinated by aortic puncture. When unclotted blood was required 300 units of heparin were injected first. 8 to 10 ml. per rat was the usual volume of blood obtained by this means. When blood was required in small amounts as for antibody estimation it was drawn by cardiac puncture using a $1\frac{1}{4}$" 23 gauge needle with the animal under ether anaesthesia. Plasma was obtained from heparinised blood by centrifugation, and serum from clotted blood after allowing 18 to 20 hours for clot retraction to occur.

b) Erythrocytes

Preliminary efforts to separate erythrocytes from leucocytes by repeated simple centrifugation and discarding of the buffy coat showed that a pure erythrocyte preparation could not be achieved by this means. The lowest degree of contamination by leucocytes was 125,000 cells in erythrocytes derived from 1 ml. of whole blood. Subsequently virtually pure erythrocyte

preparations were obtained by means of an adaptation of Harris's modification (Harris and Ukaejiofo, 1969) of Boyum's Isopaque-Ficoll technique (Boyum, 1968).

Ficoll-Triosil Technique for obtained either lymphocytes or erythrocytes:-

a) Whole blood is centrifuged and the plasma is discarded.

b) The cell mixture is passed through nylon wool packed in the barrel of a 2 ml. syringe to remove granulocytes and platelets.

c) The cell containing filtrate is carefully layered onto the surface of a tube containing 5 ml. of the mixture of Ficoll (Pharmacia) 24 parts of a 9% solution, and Triosil 75 (Glaxo) 10 parts of a 34% solution. This is centrifuged at 400 g for 20 minutes.

d) The resultant tube has a pale band of pure lymphocytes near the surface. Below this lies a broad interface of Ficoll-Triosil mixture, and at the bottom of the tube erythrocytes are present (Plate 26). If the lymphocytes are required the lymphocyte band is removed to a clean tube, resuspended in Dulbecco's solution

Plate 26: Separation of lymphocytes from peripheral blood using Boyum's (1968) Isopaque-Ficoll technique. The erythrocytes at the bottom of the tube are separated from the discrete band of lymphocytes by a broad interface of Ficoll-Triosil mixture.

and is centrifuged at 600 g for 5 minutes, and the supernatant is discarded. The lymphocytes are then resuspended. Using the technique to obtain pure erythrocytes the degree of contamination by leucocytes was less than 500 cells in erythrocytes derived from 1 ml. of whole blood.

c) Spleen cells

These cells are satisfactorily separated by the above technique. The spleen is removed, cut into fragments and homogenised. The homogenate is suspended in Dulbecco's solution and is passed through a gauze filter to remove large fragments. The homogenate is layered onto the Ficoll-Triosil mixture.

d) Thoracic duct lymphocytes

Thoracic duct cannulations were carried out by N.F. Anderson, Department of Surgery, University of Edinburgh Medical School, according to the technique of Bollman, Cain and Grindlay (1948).

Special Instruments

a) Nylon cannula 0.75 mm. internal diameter (Portland Plastics No. 1 Flex).*

b) Unmounted 17 gauge needle through which the cannula can be threaded.

c) Metal cannula moulder** comprising a curved groove and cover which serves to place a permanent curve on the cannula during boiling.

d) Ligature introducer (Anderson, 1961).

e) Cannula forceps.

f) Hook. Modified from a Gillies skin hook.

g) Glass dissecting rods bent near the tip in a bunsen flame.

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* Portland Plastics Limited, Hythe, Kent, England.

** Designed and made by N.F. Anderson.

Preparation of the rat

The animal is anaesthetised with open ether using a nose-cone and is fastened in the supine position to an operating board by means of rubber leg bands. The left subcostal region is presented by drawing the left upper limb over towards the right side of the chest, and the flank is shaved.

Technique

A 4 cm. left subcostal incision is made which divides skin and muscle and enters the abdominal cavity. The intestines and the left kidney which requires to be mobilised are packed medially with a swab, and the upper aspect of the incision is retracted. Dissection is carried medially to expose the aorta with the thoracic duct lying on its lateral aspect. Two ligatures are passed round the duct using an Anderson introducer (Anderson, 1961). The cannula is threaded through a 17 gauge needle which is passed through the abdominal wall below the incision.

A bevel is cut near the 'U' of the cannula which is primed with heparin. The sharpened Gillies hook is used to pierce the duct between the ligatures and also

serves to elevate the near-side of the duct at the point of entry, facilitating introduction of the cannula. The ligatures are then tied round the cannula to retain it in position. The wound is closed with silk in 2 layers.

Collection of lymph

When the rat approaches consciousness it is placed in a restraining cage originally designed by Bollman (1948) and modified by N.F. Anderson. The rat is given 10% glucose-saline to drink, and receives a normal laboratory rat diet. Blockage of the cannula can be cleared by the passage of a wire stilette. The lymph is collected in a flask which stands among ice chips.

6. ANTIBODY DETECTION

In some experiments an assessment of alloantibody response to donor strain antigen was required. For this purpose a microcytotoxicity test was carried out as described by Gelasthorpe and Doughty (1971). A mixture of 50% guinea pig and 50% rat complement was used. The same guinea pig complement pool was used throughout. Rat complement was taken from the animal supplying the lymphocytes. There was found to be no change in titre with this combination over guinea pig complement alone. However the mixture reduced the natural toxicity of guinea pig serum for rat cells to a negligible level, thus making interpretation easier.

a) Microcytotoxicity test

The sera to be tested are diluted serially in 1 ml. of Dulbecco's solution and 2 µl. of each dilution is transferred to the test plate under liquid paraffin. Peripheral blood lymphocytes are used as target cells and are harvested by Harris's modification (Harris and Ukaejiofo, 1969) of Boyum's Isopaque-Ficoll technique (Boyum, 1968). Two thousand cells in 1 µl. of Dulbecco's

solution are added to each well on the plate. After 30 min., incubation at 37°C, 1 µl. of complement (50% guinea pig, 50% rat) is added and a final 30 min. incubation is begun. The wells are aspirated with a fine glass capillary tube before staining for 10 min. with 1 µl. of 0.5% Trypan Blue. 1 µl. 1% bovine serum albumin in Dulbecco's solution is then added followed by 1 µl. 5% acetic acid as a fixative. The last dilution having 50% or greater cytotoxicity is considered the end point.

b) Serum fractionation

In this technique aliquots (0.5 ml.) of serum are fractionated at room temperature (20°C) on a chromatographic column (dimensions 30 x 2.5 cm.), containing Sephadex G200 (particle size 40-120) equilibrated in phosphate buffered saline (pH 7.2, 0.06 M containing 0.15 M sodium chloride and 0.003 M sodium azide). Upward flow elution with the buffer is maintained by peristaltic pump at 5.5 ml/hr. and the column effluent is monitored at 254 mµ. in a Uvicord 1 absorptiometer. The fractions (volume = 1 ml.) are pooled into the classical 19S, 7S and 4.5S fractions and a 10S fraction

which was located in the trough between the 19S and 7S peaks. The pools obtained are concentrated to twice the original serum volume by ultrafiltration at $4^{o}C$ through 8/32 dialysis tubing. Prior to cytotoxicity testing the samples are repeatedly dialysed (3 times) at $4^{o}C$ against a large volume (250 times) of phosphate buffered saline to remove the sodium azide preservative.

7. ADMINISTRATION OF CELLS AND PLASMA OR SERUM TO RECIPIENTS

Cells were administered suspended in Dulbecco's solution. When intravenous administration was required it was carried out under direct vision into the femoral vein via a small skin incision. This method was used in preference to percutaneous injection in order to ensure complete intravenous administration.

C H A P T E R III

Techniques associated with heterotopic heart transplantation

1. EVALUATION OF THE TECHNICAL RESULTS OF HETEROTOPIC HEART TRANSPLANTATION IN THE RAT

a) Operative mortality (Table 1)

Of 200 rats subjected to transplantation the number of deaths during, and for 30 minutes following operation was 19 (9.5%). Rats receiving retransplanted hearts are not included in this series as retransplantation is attended by a high rate of technical failure. Some of the deaths attributed to anaesthesia occurred in animals with pulmonary infections, and it was found that such infection lowered tolerance to anaesthesia. On two occasions some nembutal was probably inadvertently injected intravascularly producing sudden anaesthetic death. In 10 instances haemorrhage from the suture line contributed to death, and in each case this was due to faulty technique.

CAUSE OF DEATH	NO. OF RATS
Anaesthesia	8
Haemorrhage	10
Pulmonary infection	1

TABLE 1: Causes of operative deaths among 200 rats receiving heart transplants.

b) Post-operative mortality (Table 2)

There were 25 animals in this group (12.5% of all transplants). Rats dying from 30 minutes after the end of transplantation are included. The death of some rats which occurred over 2 weeks post-operatively may not be attributable to the procedure at all, but as no cause for their deaths was found they are included in the group. Among animals sacrificed were recipients of poorly functioning heart transplants.

CAUSE OF DEATH	NO. OF RATS	DAYS AFTER OPERATION
Sacrificed	4	1,1,1,6
Wound infection	2	3,12
Unknown cause	19	4,5,6,7,8,9,11,12, 14,15,24,25,60,88

TABLE 2: Post-operative causes of death among 200 rats receiving heart transplants.

2. INTRAPERITONEAL NEMBUTAL ANAESTHESIA. AN EVALUATION OF THE SITE OF DEPOSITION OF INJECTIONS USING COLLOIDAL CARBON

This brief study was undertaken in view of occasional variable results of intraperitoneal injections of nembutal. In some instances rats appeared to be little affected by adequate doses of the drug, and on other rare occasions anaesthesia developed within a few seconds of injection.

Experimental Plan

Animals. 100 F_1 hybrid rats weighing 100-200 G which were to be sacrificed in the course of a separate experiment were used.

Technique of injection. Injections were carried out through a 25 gauge needle with the anaesthetised animal held supine. The abdominal wall was not "tented" with the fingers of the other hand, and the needles were inserted with a jabbing motion midway between xiphisternum and pubis. 0.1 ml. of colloidal carbon was injected into each rat, and laparotomy was carried out on the dead animal within 5 minutes.

Results

It can be seen from Table 3 that only 77% of injections results in intraperitoneal deposition of the colloidal carbon. In 14% injection occurred into some part of the gastro-intestinal tract.

SITE OF INJECTED CARBON	NO. OF RATS
Intraperitoneal	77
Retroperitoneal	8
Caecum	7
Stomach	5
Small bowel	2
Part intraperitoneal part intravascular	1

TABLE 3: Site of deposition of colloidal carbon given "intraperitoneally".

3. DISCUSSION

Throughout the study a single surgeon (the author) performed all of the transplant operations which number approximately 500. While time-consuming such a situation allows an accurate assessment of the technical results to be made. In the early stages of the project strict standardisation of the technique was not achieved and the duration of operation was irregular, often being in excess of one hour. With subsequent experience a constant operation time of 30 minutes was achieved. For this reason an assessment was made of the last 200 procedures only.

More than half of the operative deaths were potentially avoidable. Among rats dying from haemorrhage faulty anastomotic technique was the commonest factor. In some cases haemorrhage occurred from coronary vessels which had been pierced inadvertently with the needle.

Some of the anaesthetic deaths were potentially avoidable, but others to which respiratory infections contributed could not have been anticipated.

The operative mortality may be compared with a series reported by Tinbergen (1971) in which rat renal

allografts were transplanted. A per-operative mortality of 24% was recorded in a series of 282 animals. Renal transplantation takes longer to perform than does cardiac transplantation, and the higher mortality may in part be related to this. However the major cause of death was noted as "uncontrollable haemorrhage". Tinbergen did not use the operating microscope and it seems possible that the present study achieved technically better anastomoses with the aid of the Zeiss binocular microscope.

Unknown factors caused the majority of post-operative deaths in the present study, but it is probable that lung infections may have contributed to the deaths. The inbred rats used are prone to such infection and rats which have not had operations are frequently affected. Not all of the animals dying post-operatively could be examined post-mortem as cannibalism among the inmates of a cage was not uncommon.

Among rats sacrificed were recipients of poorly functioning grafts. The causes of such failures included air embolism of the coronary vessels, and coronary sinus occlusion due to a poorly positioned caval ligature.

The low incidence of wound infection can probably be accredited largely to innate resistance of rats to such infection, though careful technique must have contributed also.

The second experiment concerning the site of deposition of intraperitoneal injections was suggested by occasional variable results of nembutal given by this route. Twenty-three per cent of injections did not results in intraperitoneal deposition. This is relatively unimportant when administering nembutal but agents such as antilymphocytic serum might be rapidly inactivated when injected into the stomach or small bowel. It is possible that the use of a wide gauge needle might result in less easy penetration of the bowel wall.

C H A P T E R IV

Demonstration of the phenomenon of prolongation of allograft survival time following pre-treatment of recipients with donor strain blood

DEMONSTRATION OF THE PHENOMENON OF PROLONGATION OF ALLOGRAFT SURVIVAL TIME FOLLOWING PRE-TREATMENT OF RECIPIENTS WITH DONOR STRAIN BLOOD

A report by Marquet, Heystek and Tinbergen (1971) demonstrated prolongation of survival time of rat renal and cardiac allografts following pre-treatment of the recipient with donor strain blood. The present experiments were designed to assess whether confirmation of this work could be obtained with regard to cardiac and skin allografts using a different strain combination.

1. Survival time of A2/1 cardiac allografts in untreated Hooded recipients and in recipients pre-treated with physiological saline

Experimental design

16 Hooded rats received A2/1 cardiac allografts without pre-treatment. A further 9 animals were pre-treated with 2 ml. physiological saline given intra-venously 14 days before transplantation. In both groups the graft survival time was assessed.

Results

Table 4 shows that the mean graft survival time in

I.V. Treatment on Day -14	No. of Rats	Allograft Survival (days)	
		Individual Values	Mean ± SE
NIL	16	10,8,14,31,14,11, 12,19,13,12,14, 14,14,15,14,11	14 ± 1.3
0.9% sodium chloride soln. 2 ml.	9	20,11,9,19,9,14, 14,14,18	14 ± 1.4
Donor strain blood 2 ml.	15	87,63,184,17*,71, 108,36,32,37,9*, 65,14*,22*,50,65	57 ± 11.6

Table 4: Cardiac allograft survival in untreated recipients, in recipients pre-treated with sodium chloride solution and in recipients pre-treated with donor strain blood 14 days before transplantation.

By Student's t test the statistical significance of the difference between the mean of the untreated group and that pre-treated with donor strain blood is $P < 0.001$.

* Died of intercurrent disease with active transplant.

untreated Hooded recipients was 14 ± SE 1.3 days. The earliest rejection took place on Day 8 and the latest on Day 31 after transplantation. A similar survival time occurred in the group pre-treated with saline (14 ± SE 1.4 days), and in this group all grafts were rejected between 9 and 20 days.

2. Survival time of A2/1 cardiac allografts in Hooded recipients pre-treated with donor strain blood

Experimental design

15 prospective Hooded recipient rats each received 2 ml. of A2/1 blood intravenously 14 days before receiving an A2/1 cardiac allograft. The blood was given within 10 minutes of exsanguination of the donor animal under direct vision into the femoral veins of the recipients. The blood donors were injected with 300 units of heparin prior to exsanguination.

Results

Table 4 shows that the mean graft survival time in rats pre-treated with donor strain blood was 57 ± SE 11.6 days. This represents a 4-fold increase in survival time as compared with untreated recipients. It is

evident that the blood pre-treatment did not produce a uniform increase in graft survival time in all recipients as there was a wide range of individual variation. The earliest rejection took place on Day 32 and the latest on Day 108 after transplantation. In no instance did indefinite graft survival occur.

3. Survival time of A2/1 skin grafts on Hooded recipients pre-treated with donor strain blood

Experimental design

The protocol of administration of donor strain blood was the same as that in the preceeding experiment. On Day 0 split-thickness skin grafts were applied as described in the section on Materials and Methods. The grafts were examined from Day +7 after application, and the time at which rejection took place was assessed. This was compared with a series reported by Anderson, James and Woodruff (1967) in this laboratory using the same strain combination.

Results

The mean skin allograft survival time in the pre-treated group was 11 days as compared with a control

time of 8 days (Table 5). The range of times of rejection was small and all grafts were rejected within a 3 day period. It is evident therefore that pre-treatment of recipients with donor strain blood has considerably less protective effect on skin allografts than on cardiac allografts.

I.V. Treatment on Day -14	No. of Rats	Allograft Survival (days)	
		Individual Values	Mean
NIL (Anderson, James and Woodruff 1967)	12	8,8,8,8,8, 8,8,8,8,8, 8,9	8
2 ml. donor strain blood	7	10,11,11, 11,12,12,12	11

Table 5: Skin allograft survival showing the effect of intravenous pre-treatment of recipients with 2 ml. donor strain blood 14 days before transplantation.

4. DISCUSSION

Prolongation of survival time of rat cardiac allografts following pre-treatment of recipients with donor strain blood has been reported previously by others (Marquet, Heystek and Tinbergen, 1971; Jenkins and Woodruff, 1971). Marquet and his associates also demonstrated similar protection of renal allografts and Marino and Benain (1958) reported prolongation of survival of skin grafts following pre-treatment of the recipient with donor strain blood.

Pre-treatment of recipients with donor strain spleen cells and bone marrow cells has been shown by Stuart, Saitoh and Fitch (1968) and by Ockner, Guttman and Lindquist (1970a) to induce prolonged survival of renal allografts in rats.

Prolongation of graft survival time by similar means has also been demonstrated in species other than the rat. Protection of skin allografts in mice has been reported by Shapiro, Martinez and Good (1961), Billingham , Brent and Medawar (1956) and Guttman and Aust (1961), and in rabbits by Billingham and Sparrow (1955), Stark, Brownlee and Grunwald (1958) and Stark

and Dwyer (1959).

Protection of canine renal allografts following pre-treatment of recipients with donor spleen cells has been reported (Halasz, Orloff and Hirose, 1964; Zimmerman, Busch, Stuart and Wilson, 1965; Linn, 1966). Others using pre-treatment schedules of lymphoid cells or blood have not shown alteration of survival time of such grafts (Calne, Davis, Medawar and Wheeler, 1966). Wilson Rippin, Dagher, Kinneart and Busch (1969) were unable to prolong survival time of such grafts using pre-treatment with donor antigen alone but the addition of minimal amounts of immunosuppressives after transplantation was found to be effective.

Pre-treatment of human skin graft recipients with donor blood has been shown by Peer, Bernhard, Walker, Bagli and Christiensen (1957) to induce some prolongation of time of graft survival.

Experiment 1 in the present study shows that the mean survival time of cardiac allografts in the strain combination used was 14 days. There was some individual variation as compared with the survival of skin grafts in the same strain combination as reported by Anderson, James and Woodruff (1967). Pre-treatment

of recipients with 2 ml. physiological saline 14 days before transplantation did not affect graft survival time.

Experiment 2 shows that pre-treatment of recipients with 2 ml. donor strain blood 14 days pre-operatively produced marked prolongation of cardiac allograft survival time. The individual variation of survival time in untreated rats was accentuated following blood pre-treatment, and if rats which died of intercurrent disease are excluded, the survival times ranged from 32 to 184 days. It appears that all animals responded to some degree and there was a moderately even scatter of times of graft survival. It therefore seems unlikely that more than one population of rats exists within the group. Indefinite survival of cardiac allografts observed by Marquet, Heystek and Tinbergen (1971) following similar pre-treatment of recipients did not occur in the present experiment.

Experiment 3 shows that only modest prolongation of skin graft survival was produced by blood pre-treatment as compared with cardiac grafts. Modification of rejection of skin allografts is recognised to be more difficult than that of organ allografts, and Marquet,

Heystek and Tinbergen (1971) did not alter skin graft survival using a similar pre-treatment schedule to the present experiments. Heslop (1966) however reported some prolongation of survival of rat skin allografts following pre-treatment of the recipients with non-viable donor strain antigen. Viable cells were found to induce accelerated rejection.

The mechanism by which graft survival time is prolonged in these experiments has not been elucidated, but immunological enhancement is a possibility. The experiments in Chapter V are aimed at proving the immunological basis of the phenomenon.

C H A P T E R V

Demonstration of the immunological specificity of prolongation of survival time of cardiac allografts following pre-treatment of the recipient with donor strain blood

DEMONSTRATION OF THE IMMUNOLOGICAL SPECIFICITY OF PROLONGATION OF SURVIVAL TIME OF CARDIAC ALLOGRAFTS FOLLOWING PRE-TREATMENT OF THE RECIPIENT WITH DONOR STRAIN BLOOD

The aim of this experiment was to evaluate the specificity of the prolongation of graft survival time demonstrated in Experiment 2, page 55. Initially Sprague-Dawley rats were used as "third party" animals. Subsequently Lewis rats were employed.

Experimental Plan

Group 1. Hooded recipients of A2/1 cardiac allografts were pre-treated i.v. with 2 ml. Sprague-Dawley blood 14 days before transplantation.

Group 2. Hooded recipients of A2/1 cardiac allografts were pre-treated i.v. with 2 ml. Lewis blood 14 days before transplantation.

Results

Table 6 shows the effect on graft survival time of pre-treatment of prospective recipients of cardiac allografts with "third party" blood of two different origins.

Pre-treatment with Sprague-Dawley blood resulted in an intermediate but highly significant degree of prolongation of survival time (27 ± SE 2.6 days) as compared with untreated controls (14 ± SE 1.3 days; Table 4).

The survival time of grafts in rats pre-treated with Lewis blood (13 ± SE 2.6 days) howeverdid not exceed that of untreated controls.

Group	No. of Rats	I.V. Treatment on Day -14	Allograft Survival (days)	
			Individual Values	Mean ± SE
Controls*	16	NIL	10, 8,14, 31,14,11, 12,19,13, 14,12,14, 14,14,11, 15	14 ± 1.3
1	6	Sprague-Dawley blood. 2 ml.	27,31,36, 17,28,27	27 ± 2.6
2	8	Lewis blood. 2 ml.	8,12,12, 12,10,10, 31,10	13 ± 2.6

Table 6: Cardiac allograft survival showing the effect of pre-treatment of recipients with blood from rats of third party strain.

By Student's t test the statistical significance of the difference between group means are as follows.

Controls : 1 $P < 0.001$
Controls : 2 N.S.

* From Table 4.

DISCUSSION

The failure of pre-treatment with Lewis blood to affect the survival time of A2/1 allografts suggests that the phenomenon demonstrated in the previous experiments is immunologically specific. The Lewis strain carries the AgB_1 antigen and the A2/1 the AgB_2 antigen. The reason for the intermediate degree of increased survival time produced by Sprague-Dawley blood pre-treatment is in doubt but the most likely explanation is the sharing of some histocompatibility antigens between these animals and A2/1 strain rats.

Demonstration of the specificity of prolongation of graft survival time shown in previous experiments furthers the suggestion that enhancement is responsible for the effect. Later experiments are aimed at identification of antibodies in donor cell treated recipient strain rats, and assessment of immunocompetence of thoracic duct lymphocytes of recipient rats following such treatment.

CHAPTER VI

Identification of the factor in donor strain blood responsible for producing prolongation of cardiac allograft survival time in recipient strain animals

IDENTIFICATION OF THE FACTOR IN DONOR STRAIN BLOOD RESPONSIBLE FOR PRODUCING PROLONGATION OF CARDIAC ALLOGRAFT SURVIVAL TIME IN RECIPIENT STRAIN ANIMALS

The aim of this experiment was to assess the effect on cardiac allograft survival of pre-treatment of recipients with donor strain plasma, erythrocytes and leucocytes given separately in approximately those amounts present in 2 ml. of whole blood. Initial studies showed that this volume of blood contained on average 1.3 ml. plasma and 0.7 ml. of packed erythrocytes. The average peripheral blood white cell count in 12 A2/1 strain male rats was 9.192 WBC/cu.mm. 88% of these were lymphocytes. Thus 2 ml. of whole blood would be expected to contain 18×10^6 lymphocytes. Thoracic duct lymphocytes were used in preference to peripheral blood cells owing to the ease with which the cells are harvested.

As previously mentioned the initial technique of simple centrifugation for isolation of erythrocytes resulted in significant contamination with leucocytes (1.7×10^6 leucocytes per batch of erythrocytes), and a virtually pure erythrocyte preparation was subsequently

obtained by Harris's modification (Harris and Ukaejiofo, 1969) of Boyum's Isopaque-Ficoll Technique (Boyum, 1968). Two subgroups are therefore present in Group 2; 2a received "contaminated" erythrocytes, and 2b received virtually pure erythrocytes.

Experimental Plan

3 groups of recipient strain rats were set up.

Group 1: Pre-treated i.v. on Day -14 before transplantation with 1.3 ml. A2/1 strain plasma.

Group 2a: Pre-treated i.v. on Day -14 before transplantation with 0.7 ml. A2/1 strain packed erythrocytes (plus "contamination" with 1.7×10^6 leucocytes).

Group 2b: Pre-treated i.v. on Day -14 before transplantation with 0.7 ml. A2/1 strain packed erythrocytes (virtually no leucocytes).

Group 3: Pre-treated i.v. on Day -14 before transplantation with 16×10^6 - 26×10^6 A2/1 strain thoracic duct lymphocytes.

In all groups the cardiac allograft survival time was assessed.

Results

Table 7 shows that the mean graft survival time in recipients pre-treated with donor strain plasma and with pure erythrocytes was 16 ± SE 2.1 days and 17 ± SE 2.0 days respectively. There is no statistically significant difference between these group means and that of rats receiving no pre-treatment (Table 4). This shows that neither donor strain plasma nor erythrocytes are capable of inducing prolongation of graft survival.

Group 3 rats which received donor strain thoracic duct lymphocytes had a mean graft survival time of 47 ± SE 8.0 days. The difference between this group mean and that of rats pre-treated with donor strain blood (57 ± SE 11.6 days; Table 4) is not statistically significant.

Group 2a rats which were pre-treated with 1.7×10^6 peripheral blood leucocytes in addition to erythrocytes showed an intermediate degree of prolongation of graft survival (35 ± SE 4.9 days). As with blood and with thoracic duct lymphocyte pre-treatment there was considerable individual variability of times of survival.

Group	No. of Animals	Pre-Treatment on Day -14 I.V.	Allograft Survival (days)	
			Individual Values	Mean ± SE
Controls*	16	NIL	10, 8,14, 31,14,11, 12,19,13, 14,12,14, 14,14,11, 15	14 ± 1.3
1	10	Donor strain plasma 1.3 ml.	22,25,27, 10,17,13, 18,11, 7, 14	16 ± 2.1
2a	12	Donor strain packed erythrocytes 0.7 ml. (+ 1.7×10^6 WBC)	16,22,25, 21,16,23, 41,64,56, 45,56,40	35 ± 4.9
2b	6	Donor strain pure packed erythrocytes 0.7 ml.	12,15,25, 19,13,15	17 ± 2.0
3	13	Donor strain thoracic duct lymphocytes (16×10^6 - 26×10^6)	71,33,37, 87,42, 9, 15,64,103, 19,28,62, 41	47 ± 8.0

Table 7: Cardiac allograft survival. Effect of pre-treatment of recipients with donor strain plasma, erythrocytes, and thoracic duct lymphocytes.

By Student's t test the statistical significance of the differences between the group means were as follows:-

Controls : 1 : 2b N.S.
Controls : 3 $P < 0.001$
Controls : 2a $P < 0.001$

DISCUSSION

These results show the ability of donor strain thoracic duct cells to initiate increased times of survival of cardiac allografts when given intravenously 14 days before transplantation. The prolongation of survival is comparable to that produced by whole blood pre-treatment in Experiment 2, page 55. (Mean survival time = 57 $\pm$ SE 11.6 days). By Student's t Test the difference is not significant. Group 3 rats also show considerable variation in responsiveness to the thoracic duct lymphocyte pre-treatment as occurred with whole blood pre-treatment. These findings suggest that the ability of whole blood to affect graft survival time may depend upon its lymphocyte content.

Stark and Dwyer (1959) however found that the erythrocyte content of donor blood was responsible for initiating prolongation of survival of rabbit skin grafts. Moreover they found that blood leucocytes were without effect. This is contrary to the findings of the present experiment and it is possible that in the rabbit histocompatibility antigens are represented on erythrocytes. The species difference may also be a

critical factor.

The intermediate degree of prolongation of survival time of grafts in rats which received erythrocytes together with leucocytes (Group 2a) suggests that the accompanying leucocytes are responsible for the phenomenon, and that quite small numbers of donor strain cells are effective though to a lesser degree than the larger number of cells used in Group 3. By inference therefore an optimum number of such cells exists which would produce maximum increase in graft survival time. An assessment of this probability is included among experiments in Chapter VII.

C H A P T E R VII

Assessment of optimum conditions for pre-treatment of recipients of cardiac allograft using donor strain spleen cells

ASSESSMENT OF OPTIMUM CONDITIONS FOR PRE-TREATMENT OF RECIPIENTS OF CARDIAC ALLOGRAFTS USING DONOR STRAIN SPLEEN CELLS

The aim of the following experiments was to assess the optimum number of donor strain cells, the optimum route of administration, and the optimum time before transplantation. Spleen cells were used instead of thoracic duct lymphocytes as their preparation was found to be simpler and less time-consuming than thoracic duct cannulation.

1. Assessment of optimum number of donor strain spleen cells which induce maximum increase in cardiac allograft survival

Experimental Plan

6 groups of recipient strain animals were set up. These were injected i.v. with 10^2, 10^3, 10^4, 10^6, 10^7, 10^8 donor strain spleen cells 14 days before transplantation, and cardiac allograft survival time was assessed.

Results

Table 8 shows that pre-treatment of recipients with 10^6 or more donor strain spleen cells 14 days before transplantation induced marked prolongation of graft survival time. The maximum effect occurred with 10^7 cells (60 ± SE 12.3 days). This represents a four-fold increase over graft survival time in untreated recipients (14 ± SE 1.3 days; Table 4). Less marked prolongation of survival occurred in the group which received 10^6 cells (24 ± SE 3.9 days). Pre-treatment with 10^4 or fewer cells had no significant effect on survival of the grafts.

2. Assessment of the optimum route of injection of donor strain spleen cells on cardiac allograft survival

Experimental Plan

2 groups of recipient animals were set up. 10^7 donor strain spleen cells were given 14 days before transplantation intraperitoneally and intradermally. The effect on cardiac allograft survival was assessed and was compared with the effect of the intravenously injected cells in the preceeding experiment.

No. of donor strain spleen cells injected I.V. on Day -14 before transplantation	No. of Animals	Allograft Survival (days)	
		Individual Values	Mean ± SE
10^2	5	13,10,14,14,14	13 ± 0.8
10^3	5	10,12,7,12*,31	14 ± 4.2
10^4	6	14,24,24,12,12,16	17 ± 2.3
10^6	7	14,25,39,25,14*, 34,14	24 ± 3.9
10^7	10	22,54,21,21,59,90, 59,41,143,90	60 ± 12.3
10^8	8	42,48,20,10,15, 33,24*,116	39 ± 12.0

Table 8: Cardiac allograft survival. Effect of pre-treatment of recipients with varying numbers of donor strain spleen cells.

By Student's t test the statistical significance of the differences between the means of the groups receiving varying pre-treatment were as follows:

No pre-treatment (Table 4) : 10^2 : 10^3 : 10^4 N.S.

No pre-treatment : 10^6 $P < 0.005$

No pre-treatment : 10^7 $P < 0.001$

No pre-treatment : 10^8 $P < 0.01$

10^6 : 10^7 $P < 0.05$

10^7 : 10^8 N.S.

* Died of intercurrent disease.

Results

These are summarised in Table 9.

The graft survival time in rats receiving intraperitoneal cells was 51 days, and in rats receiving cells intradermally it was 21 days. This is in comparison to the effect of intravenous administration in the previous experiment (60 days).

3. Assessment of optimum timing of treatment with donor strain spleen cells on cardiac allograft survival

Experimental Plan

5 groups of recipient animals were set up. 10^7 donor strain spleen cells were given i.v. on Days -150, -7, -1, 0, and +1 relative to the day of transplantation. The effect on graft survival was assessed and was compared with the effect of injection -14 days before transplantation (Experiment 1, page 65).

Results

Table 10 shows that of the time schedules used cells given 14 days before transplantation induced the greatest increase in graft survival (60 $\pm$ SE 12.3 days). Cells

Route of Administration	No. of Animals	Allograft Survival (days)	
		Individual Values	Mean ± SE
Intravenous*	10	22,54,21,21,59, 90,59,41,143,90	60 ± 12.3
Intraperitoneal	9	48,25,39,25,66, 90,66,52,52	51 ± 6.9
Intradermal	9	8,26,11,26,23, 40,12,17,24	21 ± 3.3

Table 9: Cardiac allograft survival. Effect of variations in route of injection of 10^7 donor strain spleen cells given 14 days before transplantation.

By Student's t test the statistical significance of the means of the groups receiving pre-treatment by varying routes were as follows:

Intravenous : Intraperitoneal N.S.

Intravenous : Intradermal $P < 0.01$

Intraperitoneal : Intradermal $P < 0.002$

* From Table 8.

Time of Injection (Days before Transplantation)	No. of Animals	Allograft Survival (days)	
		Individual Values	Mean ± SE
-150	4	9,36,15,11	18 ± 6.2
-14*	10	22,54,21,21,59,41, 143,90,90,59	60 ± 12.3
-7	8	25,44,55***,25,25, 43,28,55***	37 ± 4.7
-1	12	12,28,24,14,27,17, 29,76,76,69,50,61***	40 ± 7.1
0**	10	13,16,42,25,32,31,42, 49,57,53	35 ± 5.0
+1	5	7,12,16,16,19	14 ± 2.1

Table 10: Cardiac allograft survival. Effect of variations in timing of intravenous injection of the recipient with 10^7 donor strain spleen cells.

By Student's t test the differences between the mean of rats receiving no pre-treatment (N.P.T.) (Table 4) and of rats receiving spleen cells at varying times were as follows:

NPT : cells on day -150 NS

NPT : cells on day -14 $P < 0.001$

NPT : cells on day -7 $P < 0.001$

NPT : cells on day -1 $P < 0.001$

NPT : cells on day 0 $P < 0.001$

NPT : cells on day +1 N.S.

* From Table 8.
** Heart not yet rejected.

given during transplantation resulted in a mean survival time of 35 ± SE 5.0 days. Cells given on the day after operation were without effect, and those given 150 days beforehand induced only slight prolongation of graft survival (18 ± SE 6.2 days).

4. DISCUSSION

The results summarised in Tables 8, 9 and 10 show that of the schedules used 10^7 donor strain spleen cells given intravenously 14 days before transplantation induced the maximum increase in graft survival time. Ockner, Guttman and Lindquist (1970a) using donor strain bone marrow cells to pre-treat recipients of renal allografts also found that optimum results were achieved with 10^7 to 10^8 cells. The present experiments show that pre-treatment with less than 10^6 cells does not significantly affect graft survival. The survival time following injection of 10^7 spleen cells (60 ± SE 12.3 days) does not differ significantly from that induced by pre-treatment with blood (57 ± SE 11.6 days).

Table 9 shows that although pre-treatment given 14 days before transplantation is optimum, some

prolongation of graft survival was achieved by cells given just prior to release of the vascular clamps during the transplant operation (35 ± SE 5.0 days). However cells given on Day +1 after transplantation were ineffective, and those given 150 days beforehand produced minimal effect. The effectiveness of late administration of donor strain antigen in prolonging graft survival has been noted by others (Zimmerman, 1971; Kaliss, 1954).

The mechanism of prolongation of graft survival following pre-treatment with donor strain cells may relate to the balance of the cellular and humoral components of the immune response (Hutchin, 1968), and cellular immunity has been shown to preceed enhancement following active conditioning (Chantler and Batchelor, 1964; Kaliss and Bryant, 1958; Mitchison and Dube, 1955). Protocols of administration which favour antibody production, including that of enhancing antibody, may result in prolongation of graft survival, while those which induce a cellular immune response may cause accelerated graft rejection.

The importance of timing of pre-treatment in some systems may be related to subsidence of a cell mediated

response and development of a humoral response by the time that transplantation is performed. In the present experiments it is apparent that antigen given during transplantation prolongs graft survival, suggesting that little if any cell mediated immunity is induced by such injection in this system.

Immunological enhancement is known to be associated with the presence of 7S antibody, and Uhr (1964) has shown that a large antigenic challenge is associated with production of this class of antibody. In addition small amounts of antigen may result in 19S antibody which in some systems is capable of damaging allografts (Möller, 1966). There is no evidence in the present experiments that small cell doses resulted in graft damage, but the optimum dose of 10^7 cells may be related to 7S antibody production with consequent enhancement.

As well as the dose, timing and route of administration of the antigen, its physical form and frequency of administration may dictate whether a predominantly humoral or cellular response occurs. Antigen given in multiple small injections is more effective than when given in a single large injection (Hutchin, 1968). The viability of the antigen also

dictates to some extent the form of response (Billingham and Silvers, 1971). In general non-viable preparations give rise to more marked humoral responses. Heslop (1968) found that enhancement of rabbit skin grafts occurred following pre-treatment with non-viable donor cells, but that accelerated rejection followed the use of viable cells. There is no doubt however that different systems present different requirements regarding the pre-treatment protocol.

A point of interest arising from these experiments is the wide variations of pre-treatment protocol which are capable of inducing prolongation of graft survival. In this system therefore there is no evidence that the effect is dependent upon pre-treatment with antigen according to precisely defined parameters. Not only did accelerated rejection not occur but it was difficult not to induce at least some prolongation of graft survival except with extremes of protocol.

C H A P T E R VIII

Administration of further donor strain blood and sensitised recipient strain spleen cells to recipients of cardiac allografts previously conditioned with donor strain blood

ADMINISTRATION OF FURTHER DONOR STRAIN BLOOD AND SENSITISED RECIPIENT STRAIN SPLEEN CELLS TO RECIPIENTS OF CARDIAC ALLOGRAFTS PREVIOUSLY CONDITIONED WITH DONOR STRAIN BLOOD

The aim of this experiment was to assess whether prolongation of survival time of cardiac allografts following pre-treatment of recipients with donor strain blood could be affected by a further injection of either donor strain blood 14 days after transplantation or by sensitised recipient strain spleen cells 21 days after transplantation.

Experimental Plan

a) 11 Hooded rats each received an A2/1 cardiac allograft 14 days after an intravenous injection of donor strain blood (2 ml.). 14 days after transplantation a further similar injection of blood was given and the effect on graft survival time was assessed.

b) 7 Hooded rats each received an A2/1 cardiac allograft 14 days after an i.v. injection of donor strain blood (2 ml.). 21 days after transplantation 10^7 sensitised spleen cellswere given intravenously which were harvested

from 3 Hooded rats 8 days after application of donor strain split skin grafts (skin grafts were rejected on Day 8 after application).

Results

a) The mean survival time of cardiac allografts in this group was 63 ± SE 5.8 days (Fig. 1). At the time of reporting 4 grafts were not rejected. This exceeds the survival time in recipients pre-treated with blood but without subsequent injections (Table 4).

b) 10^7 Hooded sensitised spleen cells given intravenously on Day +21 after transplantation to conditioned Hooded recipients of A2/1 cardiac allografts were associated with a mean graft survival time of 66 ± SE 1.23 days (Fig. 2). At the time of reporting 4 grafts were not rejected. This also exceeds the mean survival time of grafts in recipients pre-treated with blood but without subsequent injections.

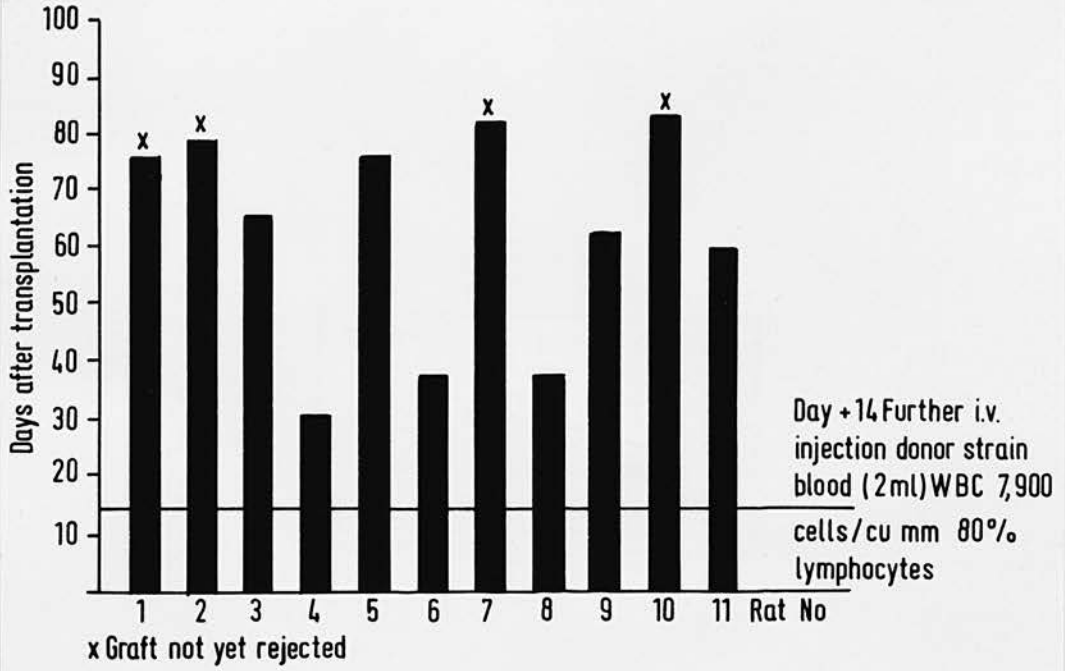

Fig. 1: Cardiac allograft survival in recipients injected with 2 ml. donor strain blood on days -14 and +14 relative to the day of transplantation.

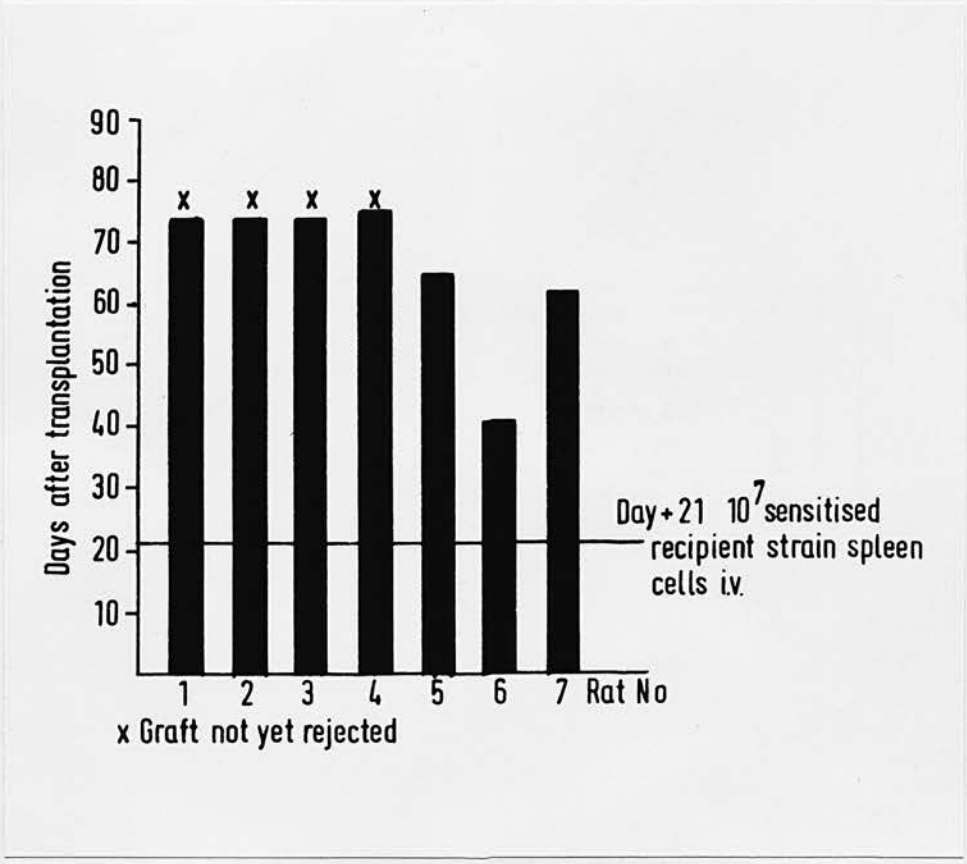

Fig. 2: Cardiac allograft survival. Recipients pre-treated 14 days before transplantation with 2 ml. donor strain blood. 10^7 sensitised recipient strain spleen cells given intravenously on day +21 after transplantation.

DISCUSSION

In this system it is evident that a further injection of donor strain blood 14 days after transplantation does not shorten graft survival time. It is however possible that in some cases the injection has induced increased graft survival over and above that produced by pre-treatment alone as 4 grafts have not yet been rejected. It is conceivable therefore that repeated injections of donor strain blood after transplantation might result in still greater prolongation of graft survival. It may be speculated that such injections may induce a prolonged humoral response including enhancing antibody, but little cellular immunity.

Experiment (b) shows that A2/1 cardiac allografts, the survival of which is prolonged by pre-treatment of the recipient with donor strain blood, are not rejected when 10^7 sensitised Hooded spleen cells are injected 21 days after transplantation. There is little doubt that the cells were sensitised as they were harvested immediately following rejection of A2/1 skin grafts. It is possible that accelerated graft rejection might have occurred if a larger cell dose had been used, but

this seems unlikely since the cells appear to be associated with a greater prolongation of graft survival than after pre-treatment with blood alone. If a state of enhancement is induced by blood pre-treatment it appears to be sufficiently strong to prevent sensitised cells from causing graft rejection, and furthermore that such cells may actually reinforce the prolongation of graft survival.

These findings are in contradistinction to those using rats made tolerant as neonates to allogeneic cells. In such rats skin allografts can be made to reject by injection of cells syngeneic with the host but from non-tolerant animals (Gowans, Gesner and McGregor, 1961; Billingham, Silvers and Wilson, 1963).

It is probable that in the present experiment the sensitised cells serve to stimulate increased antibody which further protects the graft. This further indicates that enhancement is the likely mechanism of the prolongation of graft survival.

C H A P T E R IX

Antibody studies in recipient strain rats

ANTIBODY STUDIES IN RECIPIENT STRAIN RATS

The preceeding experiments have established that the increase in graft survival time induced by pre-treatment of recipients with donor strain antigen is specific, and that the optimum schedule of antigen administration conforms to a typically enhancing schedule. This suggests that the mechanism of the effect is immunological enhancement. In order to assess this possibility the following experiments were carried out to attempt to demonstrate enhancing antibodies in recipient rats pre-treated with donor strain antigen. The possibility of inducing prolongation of graft survival time by passive transfer of serum from conditioned recipients to otherwise untreated recipients of cardiac allografts has also been examined.

1. Assessment of antibody titres

3 groups of recipient strain animals were set up and antibody titres were asessed in each at weekly intervals. Serum from 4 rats in Group a) was subjected to fractionation.

Rat No.	7 Days after I.V. Blood from Donor Strain			14 Days after I.V. Blood from Donor Strain		
	Whole Serum	7S Fraction	19S Fraction	Whole Serum	7S Fraction	19S Fraction
1	1:128	<1:8	1:64	1:32	1:32	<1:8
2	1:64	<1:8	1:16	1:8	1:8	<1:8
3	1:64	<1:8	1:64	1:8	1:8	<1:8
4	1:64	<1:8	1:32	1:16	1:8	<1:8

Table 11: Cytotoxic titres of serum fractions from 4 Hooded rats (from group a) 7 and 14 days after intravenous injection with donor strain blood.

Experimental Plan

Group a). 11 Hooded rats were each injected intravenously with 2 ml. of A2/1 blood. Serum from these rats was taken at weekly intervals and was subjected to cytotoxicity testing. As well as this, serum from 4 of the rats (selected at random) was subjected to G200 Sephadex gel filtration at 1 week and at 2 weeks after injection of donor strain blood.

Group b). 7 Hooded rats each received an A2/1 cardiac allograft as the only treatment. Serum was again taken at weekly intervals thereafter and subjected to cytotoxicity testing.

Group c). 25 Hooded rats each received 2 ml. of donor strain blood intravenously 14 days prior to receiving an A2/1 cardiac allograft. Serum was taken at weekly intervals for cytotoxicity testing.

The mean $\pm$ SD number of lymphocytes in 2 ml. of A2/1 blood injected in Groups a) and c) was $1.22 \pm 0.17 \times 10^7$ cells. The viability of the cells was not less than 98% on trypan blue exclusion.

Results

Figures 3 (Group a) and 5 (Group c) show that injection of Hooded recipients with 2 ml. of A2/1 blood i.v. induced a sudden rise in titre of antibody cytotoxic to A2/1 lymphocytes. Serum samples tested one week after injection had the highest antibody levels detected in these groups. At 2 weeks after injection the antibody level was considerably lower, and this was followed by a gradual rise in both groups. The addition of a cardiac allograft at 2 weeks in Group c) accentuated this gradual late rise in titre. Rats which received cardiac allografts without other treatment (Group b) showed a steep rise in titre between 3 and 4 weeks after grafting (Fig. 4). The mean survival time of grafts in this group was 13.4 days.

Table 11 showing results of serum fractionation from 4 rats grom Group a) shows that at 1 week after injection the cytotoxic activity was mainly in the 19S fraction. At 2 weeks it was mainly in the 7S fraction, that in the 19S fraction having subsided. No activity was found at any time in the 4.5S or the 10S fractions.

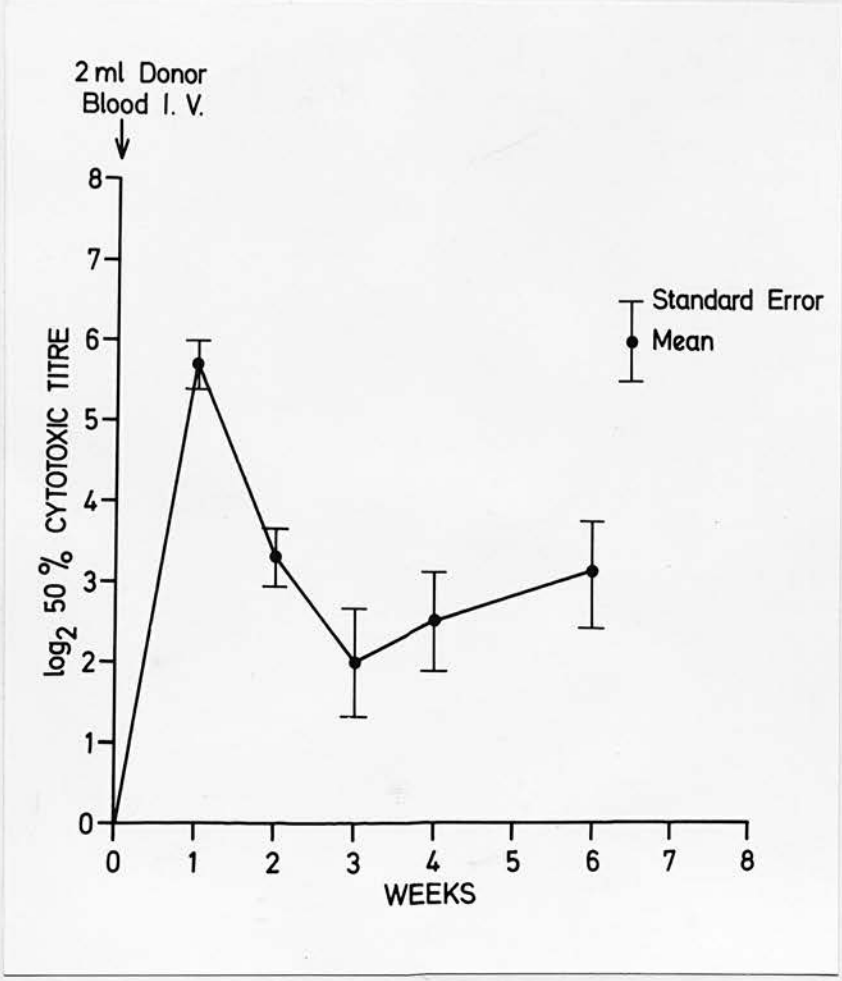

Fig. 3: Group a) rats.
Cytotoxic antibody titres in 11 Hooded rats injected intravenously with 2 ml. A2/1 blood on day 0.

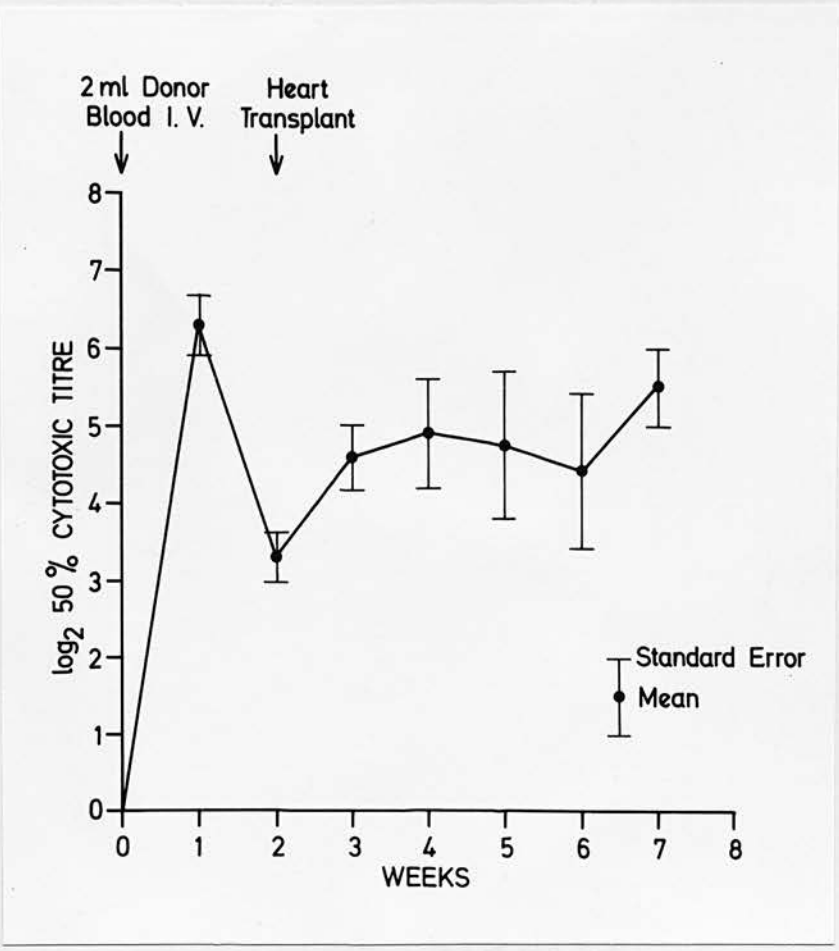

Fig. 4: Group b) rats.
Cytotoxic antibody titres in 25 Hooded rats each injected intravenously with 2 ml. A2/1 blood 14 days before receiving an A2/1 cardiac allograft. At the end of sampling at 7 weeks one rat had rejected its graft on day 45.

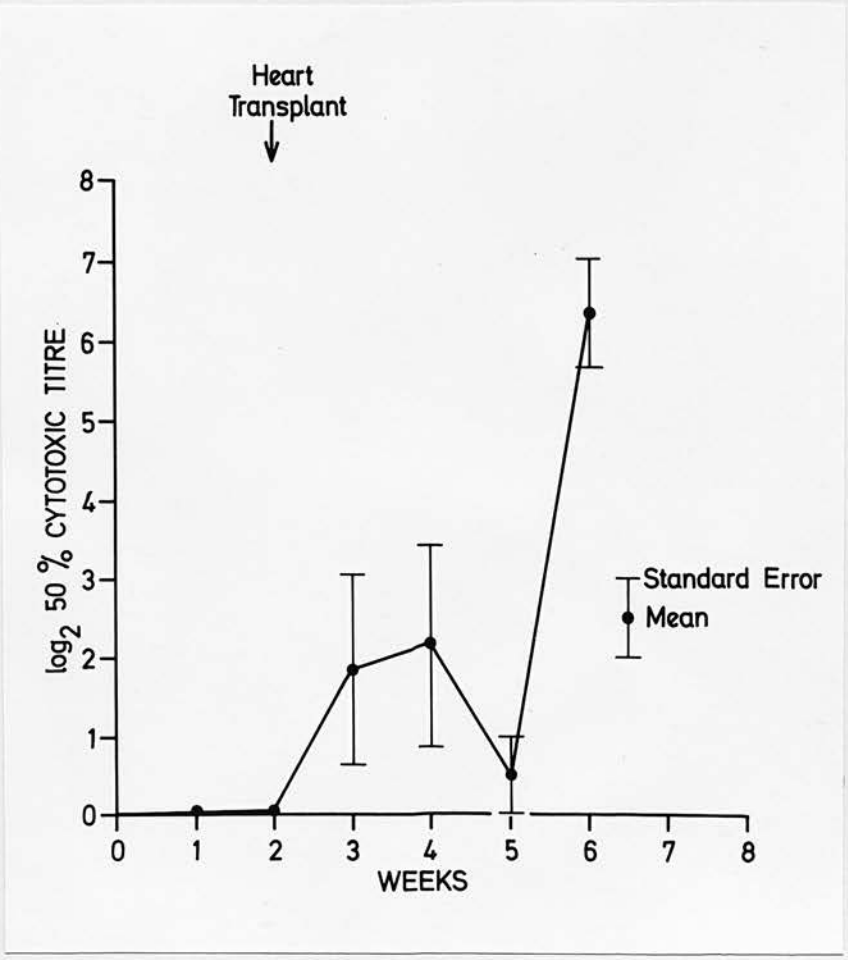

Fig. 5: Group c) rats.
Cytotoxic antibody titres in 7 Hooded rats which received cardiac allografts as the only treatment.

The mean graft survival time was 13.4 ± SE 1.4 days.

2. DISCUSSION

It is apparent that the pre-treatment schedule used in these experiments results in demonstrable cytotoxic antibodies and that such antibodies coincide with prolongation of cardiac allograft survival time. Correlation between antibody titre and graft survival in Group c) is not possible since only one graft was rejected by the end of sampling 5 weeks after transplantation. This experiment therefore while demonstrating an antibody response to injected allogeneic cells has not confirmed that this is responsible for prolongation of graft survival time. There is no evidence that accelerated graft rejection takes place in the presence of the antibody. The failure to causally relate an antibody response to prolongation of survival of allografts is common to other reports (Holl-Allen, 1971; Rapaport, Dausset, Lawrence and Converse, 1968).

Lucas, Markley and Travis (1970) however showed dissociation of rat renal allograft survival and titre of cytotoxic and haemagglutinating antibodies produced in response to antigen treatment, though antibody detectable by binding-inhibition assays was present

in all sera capable of passively transferring enhancement. Conversely Mayer, Kronman and Dumont (1965) found a direct relationship between cytotoxic antibody response to intravenously injected allogeneic thoracic duct lymphocytes and skin graft survival in rats.

Though there is wide disagreement about the role of cytotoxic antibodies in immune suppression they do without doubt cause destruction of grafts in some systems. Amos and Wakefield (1958) and Algire (1959) showed that various tumours enclosed in cell impermeable chambers were destroyed in sensitized hosts. Gorer and O'Gorman (1956) found that normal as well as tumour cells are damaged by antibody and complement. Porter (1963) reported that in man rapid rejection of renal allografts occurs when a major ABO-incompatibility exists between donor and recipient. This is presumably due to the presence of ABO antibodies in the recipient. Others have since shown that in the presence of pre-formed antibodies against donor cells, renal allografts are rejected in a hyperacute fashion (Kissmeyer-Nielsen, Olsen, Petersen and Fjeldborg, 1966; Williams, Hume, Hudson, Morris, Kano and Milgrom, 1967; Terasaki, Thrasher and Hauber, 1968; Russell, 1971).

Work by Stetson and Jensen (1960) suggests that in established homografts there may be a blood-graft barrier which protects grafts from access by potentially damaging antibody. Breakdown of the barrier as might occur during the homograft reaction might facilitate action by the antibody. Amos (1955) showed that established grafts do not take up antibody, though graft cells present within the recipients' circulation do so. Benacerraf, Biozzi and Halpern (1954) showed that circulating antibody localises at the site of injections of histamine in the skin, and Stetson and Jensen (1960) demonstrated necrosis of skin allografts in the presence of circulating cytotoxic antibody following injection of histamine into the graft. The altered vascular permeability induced by the histamine was considered to have effectively broken the blood-graft barrier.

Table 11 shows the high antibody titre which occurred one week after injection of antigen, and the cytotoxic activity at that time is shown to be predominantly in the 19S fraction. It is of some interest that cardiac allografts transplanted at that time (Table 10, page 67) still have marked prolongation of survival time. 19S antibodies are known to be more

cytotoxic than 7S antibodies (Möller, 1966; Takasugi and Hildemann, 1968) and might be expected to adversely affect graft survival. It is possible that in this model also the grafts are protected by a blood-graft barrier.

Group b) animals which received cardiac transplants as the only treatment showed a sharp rise of antibody titre between 3 and 4 weeks after transplantation. The mean survival time of these grafts was 13.4 days and no graft survived longer than 15 days. The cause of the sudden rise in titres is uncertain but it may be due to access of products of graft rejection to the hosts' immune centres. Lance and Medawar (1969) noted a rise in antibody titre following rejection of skin grafts in mice with fading tolerance to the graft.

In conclusion it is apparent that the pre-treatment schedule used in these studies gives rise to 7S antibodies which are known to be associated with enhancement (Finkelstein and Uhr, 1964; Möller and Wigzell, 1965) 2 weeks after injection of the antigen. The presence of such antibody coincides with marked prolongation of survival time of cardiac allografts. This provides strongly suggestive evidence that the immunosuppression is due to immunological enhancement.

3. Passive transfer of serum from recipient strain animals conditioned with donor strain blood

Experiment a) was designed to assess the ability of serum raised in recipient strain animals by injection of donor strain blood to produce passive enhancement in otherwise untreated recipients of cardiac allografts. The second experiment (b) was designed to assess the effect of perfusing isolated allografts with similar serum prior to their transplantation.

a) Injection into recipients of cardiac allografts

Experimental Plan

12 Hooded recipients of cardiac allografts were injected with serum from recipient strain rats which had previously been conditioned with A2/1 blood given intravenously. 3 different protocols of serum administration were used. The serum for recipients 1-5 was from rats conditioned with a single injection of blood 14 days before exsanguination. Recipients 6-15 received serum raised by conditioning injections 14 days and 7 days before exsanguination. The cytotoxic titres of these sera were assayed. Serum was injected intravenously

except for some injections to rats 13-15 which were given intraperitoneally. The schedule of administration is shown in Table 12.

The cardiac allograft survival time was assessed in all groups.

Results

Table 12 shows that injection of heart recipients with serum from Hooded donors previously conditioned with A2/1 blood did not affect graft survival time.

b) Perfusion of serum through isolated cardiac allografts prior to transplantation

Experimental Plan

Serum donors of recipient strain were conditioned with 2 ml. donor strain blood given intravenously 14 days prior to exsanguination. The cytotoxic titre of this serum was not assayed.

Immediately following removal of each heart a fine portex cannula was introduced into the aorta and was secured with a ligature. 2 ml. of serum was perfused over 5 minutes at room temperature through the coronary

Preparation of Serum			Recipient Groups		Serum doses i.v. ml. Timing relative to day of transplant							Allograft Survival (days)	
Conditioning Agent i.v.	Conditioning Regime Injection - Exsanguination Internal (days)	Cytotoxic Titre of Serum	Group of Recipients	Rat No.	-1	0[a]	+1	+2	+3	+6	+9	Individual Values	Mean ± SE
A2/1 blood 2 ml.	14	not done	a)	1	-	3	3	3	-	-	-	17	15 ± 2.0
				2	-	3	3	3	-	-	-	15	
				3	-	3	3	3	-	-	-	10	
				4	-	3	3	3	-	-	-	13	
				5	-	3	3	3	-	-	-	22	
A2/1 blood 2 ml.	14 : 7*	512		6	-	3	-	-	2	2	2	27	15 ± 2.4
				7	-	3	-	-	2	2	2	18	
				8	-	3	-	-	2	2	2	11	
				9	-	3	-	-	2	2	2	19	
				10	-	3	-	-	2	2	2	11	
				11	-	3	-	-	2	2	2	11	
				12	-	3	-	-	2	2	2	10	
A2/1 blood 2 ml.	14 : 7*	128		13	2[b]	2	-	-	2[b]	2[b]	-	13	16 ± 1.7
				14	2[b]	2	-	-	2[b]	2[b]	-	18	
				15	2[b]	2	-	-	2[b]	2[b]	-	18	
A2/1 blood 2 ml.	14	not done	b)	16	2 ml. perfused through isolated hearts prior to transplantation							13	16 ± 1.1
				17								19	
				18								16	
				19								18	
				20								14	

a = Prior to release of vascular clamps.

b = Given intraperitoneally.

Table 12: Cardiac allograft survival. Effect of passive transfer of serum from Hooded rats conditioned intravenously with 2 ml. A2/1 blood on A2/1 cardiac allograft survival in Hooded recipients.

circulation. Following removal of the ligature and cannula a cuff of aorta was excised and transplantation was carried out.

Results

It can be seen from Table 12 that perfusion of hearts prior to transplantation with serum from Hooded donors previously conditioned with A2/1 blood did not affect graft survival time (16 ± SE 1.1 days).

4. DISCUSSION

Part (a) of this experiment shows that serum from recipient strain rats conditioned with donor strain blood does not prolong graft survival time when passively transferred to allograft recipients in spite of using serum with a cytotoxic titre as high as 512 (recipients 6-12). It is possible that the antibody doses or the schedules of administration were not optimum. Haughton and Nash (1969) however showed that enhancement of mouse tumours could be achieved by injection of sub-microlitre doses of passively transferred antibody. Other studies suggest that large doses of serum must be

given in passive transfer experiments with normal tissues (Brent and Medawar, 1961; Heslop, 1966; Nelson, 1961; Nelson, 1962) since the half-life of γ globulin is quite short (Hutchin, 1968; Dixon, Talmage, Maurer and Deichmiller, 1952). It is also likely as suggested by Hutchin (1968) that a prerequisite of an enhancing serum is that it should contain antibodies against all antigenic receptors of the allogeneic graft donor. This has probably been achieved in some studies by hyperimmunisation of the serum donor. Stuart, Saitoh and Fitch (1968) and Lucas, Markley and Travis (1970) used three injections of 10^8 donor strain spleen cells the first accompanied by Freund's adjuvant to raise an allo-antiserum for passive enhancement of rat renal allografts. Studies by Stimpfling and Pizarro (1961) suggest that in mice it is necessary to hyperimmunise to induce antibodies against the weaker antigens.

Most reports of passive enhancement of organ allografts concern rat renal transplants. Stuart, Saitoh, Fitch and Spargo (1968) demonstrated passive enhancement of such grafts using allo-antiserum. French and Batchelor (1969) showed passive enhancement of rat renal allografts which was most striking when F_1

hybrid kidneys were used, and less so with allogeneic homozygous kidneys. They found that 3.5 ml. of serum with a cytotoxic titre of 1/256 given in divided doses was effective. More recently Ockner, Guttman and Lindquist (1970b) demonstrated reduced intensity of rejection of rat renal allografts after injection of recipients with a globulin fraction of serum from recipient strain animals pre-treated with donor strain bone marrow cells.

Prolongation of graft survival time in part (b) of this experiment would depend on antibody acting peripherally on the graft as suggested by Morris and Lucas (1971) rather than on the antigen processing or immune centres of the recipient. It is possible that a substantial part of the enhancing process occurs centrally, and the failure of the technique of graft perfusion to prolong survival time could be ascribed to this factor. Alternatively it is possible that the duration of contact between antibody and graft was too brief to produce an enhancing effect.

It is evident that a number of objections can be raised to the design of the two parts of this experiment and it is considered that the result does not exclude

enhancement from being the mechanism of prolongation of graft survival time demonstrated in the earlier experiments. A further investigation required is passive transfer of non-complement fixing γ globulin subclasses from serum of conditioned animals into graft recipients rather than crude serum as used in these experiments. Given sufficient serum donors much larger volumes of the active subfraction could be given.

CHAPTER X

Assessment of the immunocompetence of recipient strain rats pre-treated with donor strain blood

ASSESSMENT OF IMMUNOCOMPETENCE OF RECIPIENT STRAIN RATS PRE-TREATED WITH DONOR STRAIN BLOOD

The aim of these experiments was to determine whether or not injection of Hooded rats with A2/1 blood induces a state of tolerance in the sense used by Billingham, Brent and Medawar (1953) in which there is a state of specific non-reactivity to cells possessing foreign alloantigens.

Also assessed is whether or not injection of allogeneic blood results in temporary recruitment of antigen reactive cells from the recirculating lymphocyte pool.

The first experiment is an evaluation of the graft-versus-host assay of Ford, Burr and Simonsen (1970). In the second this assay is used to determine the immune reactivity of thoracic duct lymphocytes of Hooded rats at varying intervals after injection with A2/1 blood. The reactivity of thoracic duct lymphocytes from rats pre-treated with donor strain blood and carrying long surviving cardiac allografts is also assessed. The third experiment is a test of the ability of Hooded rats pre-treated with A2/1 blood and bearing long surviving cardiac allografts to reject donor strain split skin grafts.

1. Assessment of the graft-versus-host response in F_1-hybrid animals to lymphoid cells from untreated Hooded rats

Local graft-versus-host reactions have been produced in numerous anatomical sites such as the skin (Brent and Medawar, 1963), the kidney (Elkins, 1964) and the peritoneal cavity (Weiser, Granger, Brown, Baker, Jutila and Holmes, 1965). Efforts to quantitate local graft-versus-host reactions have resulted in a variety of techniques of differing degrees of usefulness. Ford (1967) described a local graft-versus-host assay based on the reaction of parental cells injected intradermally into F_1 hybrid animals. Its usefulness is evidently limited by difficulties with quantitation, and contamination of the inoculum with red blood cells makes it unreliable.

Elkins (1964) introduced a technique in which the cell inoculum is injected beneath the renal capsule of the F_1 hybrid recipient. On the 7th day a histological grading can be made depending on the degree of activity exhibited by the infiltrate.

Levine (1968) reported a method which evolved from attempts to inject parental spleen cells directly into surgically exposed lymph nodes. Leakage of the

inoculum from the nodes occurred and quantitation was found to be impossible. Subsequent injections were given intradermally into the footpad which gave marked weight increase of the popliteal lymph node from 6 mg. up to 185 mg. in 5-11 days. This principle was employed by Ford, Burr and Simonsen (1970) and by Hásková and Gansová (1970). A reproducible technique was developed and was used in the present experiments. Lagunoff (1969) showed by means of infusions of labelled autochthonous lymphocytes that the node enlargement is not due to trapping of circulating cells. Ehrich, Drabkin and Forman (1949) demonstrated hypercellularity and DNA increase in similar nodes.

Ford and his associates found that subcutaneous injections gave more reliable results than intradermal and they established a dose-node weight increase relationship. They found the method more reliable than others and report it as 10 times more sensitive than the Elkins' method (Elkins, 1964) as well as being more convenient to perform.

Experimental Plan

5 groups of F_1 hybrid rats were set up. Using the graft-versus-host assay of Ford, Burr and Simonsen (1970) each animal in Groups A to D received 10^7 lymphoid cells in 0.1 ml. Dulbecco's solution injected subcutaneously into the right hind footpad. Group E animals received 0.1 ml. of Dulbecco's solution alone. The animals were killed at 1 week and the popliteal nodes from both sides were weighed.

Group A: 20 F_1 hybrid rats each received 10^7 Hooded thoracic duct lymphocytes from 3 donors.

Group B: 13 F_1 hybrid rats each received 10^7 Hooded spleen cells pooled from 2 donors.

Group C: 10 F_1 hybrid rats each received 10^7 F_1 (syngeneic) thoracic duct lymphocytes from a single donor.

Group D: 10 F_1 hybrid rats each received 10^7 F_1 (syngeneic) spleen cells from a single donor.

Group E: 9 F_1 hybrid rats each received 0.1 ml. of Dulbecco's solution.

Results

These are summarised in Table 13. Injection of parental strain thoracic duct lymphocytes induced marked increases in weights of the draining popliteal nodes as compared with the contralateral nodes of F_1 hybrid rats. The combined mean of the test nodes in Group A was 55.61 mg. (51.64 - 59.88). This is approximately 10 times the mean weight of the contralateral nodes.

Injection of parental strain spleen cells induced less marked node weight increases. The mean weight of nodes in this group was 35.17 mg. (33.12 - 37.34).

Injection of syngeneic thoracic duct lymphocytes and spleen cells into F_1 hybrids induced slight node weight increases of the order of 2 mg. This effect has been noted previously by Ford, Burr and Simonsen (1970). Dulbecco's solution was without effect.

2. DISCUSSION

Table 13 shows that inoculation of F_1 animals with 10^7 parental TDLs produces marked increase in the mean weights of the draining popliteal nodes (geometric mean of the 3 subgroups in Group A is 55.61(51.64 - 59.88)).

Group	Origin of injected cells	No. of cell donors	No. of F_1 Recipients	Mean Node Weight[2]	
				Test Node[1]	Contra-lateral node
a	TDL (Hooded)	1	9	46.82 (42.78-51.24)	5.05 (4.78-5.34)
	" "	1	5	52.99 (50.65-55.43)	5.06 (4.69-5.46)
	" "	1	6	74.94 (64.74-86.74)	4.70 (4.52-4.88)
b	Spleen cells (Hooded)	2	13	35.17 (33.12-37.34)	5.05 (4.61-5.55)
c	TDL syngeneic F_1	1	10	7.94 (7.58-8.31)	5.51 (5.21-5.83)
d	Spleen cells syngeneic F_1	1	10	8.14 (7.53-8.80)	5.28 (5.03-5.53)

Table 13: Effect of injection of 10^7 lymphoid cells of parental and isogeneic origin on popliteal lymph node weights of F_1 hybrid rats.

By Student's t test the statistical significance of the difference between the means of group a) combined and group b) is $P < 0.001$.

1. Popliteal node from injected side.
2. Geometric mean (mg). The numbers in parentheses show the effect of adding or subtracting 1SE of the log mean.

This represents a ten-fold weight increase over the contralateral nodes and approximates to the findings of Ford, Burr and Simonsen (1970) when 9×10^6 parental TDLs were used.

Ford and his associates found that a saturation effect occurred when injecting a number greater than 9×10^6 parental cells and a larger number of cells did not produce a significantly greater lymph node weight. In the present study a dose of 10^7 parental cells was selected and no attempt has been made to correlate node weight response to cell dose.

Table 13, Group B shows that spleen cells induce approximately a five-fold node weight increase and therefore appear to be less active than thoracic duct lymphocytes in inducing a graft-versus-host response. The importance of the circulating small lymphocyte in initiating graft-versus-host reactions has been emphasised by Gowans, Gesner and McGregor (1961), and the relatively poor reactivity of spleen cells is probably a reflection of this. Using mice Cantor, Mandel and Asofsky (1970) showed that blood and thoracic duct lymphocytes are more than four times as active as are spleen cells in producing graft-versus-host reactions.

Injection of syngeneic thoracic duct lymphocytes and spleen cells produced small but consistent increases in node weights of about 2.0 mg. per node. This must be a non-specific effect unrelated to the trauma of injection since injection of Dulbecco's solution alone had no effect on node weights.

3. Assessment of the graft-versus-host response in F_1-hybrid animals to thoracic duct lymphocytes from Hooded rats pre-treated with A2/1 blood

Recent reports of *in vitro* and *in vivo* studies have demonstrated the selective binding or recruitment by antigen of a specific subpopulation of antigen sensitive cells (Wigzell and Anderson, 1969; Dutton and Mishell, 1967; Ada and Byrt, 1969; Ford and Atkins, 1971; Sprent, Miller and Mitchell, 1971).

Ford and Atkins (1971) showed that passaging parental strain thoracic duct lymphocytes through an irradiated F_1 hybrid rat resulted in recruitment of a subpopulation of these cells. Thoracic duct cells from the hybrid which were shown to be mostly of parental origin had a reduced graft-versus-host capability against similar F_1

hybrid animals, but not against third party hybrids. This specific unresponsiveness of passaged parental cells which was noted in the 12-36 hour lymph collection did not return at any time during the 5.5 day collection period.

Sprent, Miller and Mitchell (1971) showed also that thoracic duct lymphocytes from parental strain mice injected intravenously 1-2 days previously with F_1 hybrid spleen cells had no demonstrable graft-versus-host activity against similar F_1 hybrid animals but had normal activity against third party F_1 hybrids. The activity which was found to be absent up to 2 days was considerably augmented by day 5. They also showed that thoracic duct lymphocytes from mice 1-2 days after injection of sheep erythrocytes did not adoptively transfer immune reactivity to sheep erythrocytes in thymectomised, irradiated, marrow protected syngeneic hosts, but were normal with respect to horse erythrocytes. Cells collected at day 3 had a normal activity and cells collected at day 5 had augmented activity.

The ability of intravenous injections in some systems to produce temporary deficiency of specific antigen reactive cells in the recirculating lymphocyte

pool suggests that the blood or spleen cell pre-treatment schedule used in experiments in this thesis might produce a similar effect and that it might possibly be linked to programming of cells to produce enhancing antibody.

The aims of this experiment therefore were:-

a) To assess whether or not the recirculating pools of lymphocytes of Hooded rats become temporarily deficient in cells specifically reactive to the antigen in A2/1 blood after an intravenous injection of such blood.

b) To determine whether pre-treatment with donor strain blood alters the graft-versus-host reactivity of thoracic duct lymphocytes of such rats 14 days after injection (i.e. at the optimum time for heart transplantation).

c) To assess the graft-versus-host reactivity of thoracic duct lymphocytes of rats which have been pre-treated with donor strain blood and which are carrying long surviving cardiac allografts.

Experimental Plan

Hooded thoracic duct lymphocytes were harvested at varying intervals after intravenous injection of the donors with 2 ml. A2/1 blood. The first collection began at the time of injection of the blood, and the last was on day 14 after injection. Two or more donors contributed to each pool of thoracic duct cells. Eight cell pools were harvested. A further pool of cells was provided by a Hooded rat pre-treated with donor strain blood 14 days before receiving a donor strain cardiac allograft. The graft was still active 21 days after transplantation when thoracic duct cannulation was performed.

A graft-versus-host assay of each pool was carried out in F_1 hybrid rats using the method described by Ford, Burr and Simonsen (1970). Each F_1 hybrid was injected in one footpad and 10^7 cells in 0.1 ml. Dulbecco's solution and both test and contralateral popliteal nodes were weighed at one week.

Results

Table 14 shows the popliteal node weight increases in F_1 hybrids following injection of thoracic duct lymphocytes harvested from Hooded rats at varying intervals after pre-treatment with A2/1 blood. No cell pool induced a graft-versus-host reaction, as assessed by increase in weight of the popliteal nodes, which was significantly different from that produced by cells from untreated Hooded rats (Table 13, Group A).

Table 14 also shows that thoracic duct lymphocytes from a pre-treated Hooded rat bearing a long surviving A2/1 cardiac allograft also had normal graft-versus-host reactivity in F_1 hybrid rats.

It therefore follows that no recruitment of antigen reactive cells was demonstrated in response to injection of the thoracic duct cell donors with allogeneic blood. It is evident that the injection of blood, shown in previous experiments to prolong cardiac allograft survival, does not induce a state of immunological tolerance. Pre-treated rats carrying long surviving cardiac allografts are also shown to be immunologically competent.

Time of collection of TDLs after injection of allogeneic blood (hours)	No. of TDL Donors	No. of F_1 Hybrid Recipients	Mean Node Weight[2]	
			Test Node[1]	Contra-lateral node
0-4	2	5	75.65 (61.93-92.40)	5.25 (4.97-5.54)
4-6	2	5	60.41 (55.61-65.63)	5.28 (5.08-5.50)
6-10	2	5	58.46 (52.57-65.00)	5.25 (4.79-5.76)
14-18	3	8	69.46 (64.99-74.25)	5.64 (5.34-5.96)
18-22	3	9	69.99 (68.31-71.71)	5.76 (5.51-6.01)
24-36	2	7	62.48 (59.54-65.58)	5.32 (5.02-5.63)
24-48	2	29	72.90 (70.39-75.49)	5.53 (5.35-5.71)
14 days (24 hr. collection)	3	45	56.80 (54.88-58.80)	5.41 (5.26-5.56)
35 days after blood pre-treatment, and 21 days after heart transplantation. (24 hr. collection)	1	5	57.54 (51.12-64.76)	6.00 (6.36-5.67)

Table 14: Effect of injection of 10^7 Hooded thoracic duct lymphocytes (harvested at varying intervals after intravenous injection with A2/1 blood) on popliteal lymph node weight of F_1 hybrid rats. Effect of cells from a pre-treated Hooded rat carrying a long surviving cardiac allograft.

By Student's t test the difference between the means of group a (Table 13) and that of rats injected with thoracic duct lymphocytes taken 0-4 hours after injection of allogeneic blood is not statistically significant.

1. Popliteal node from injected side.
2. Geometric means (mg). The numbers in parentheses show the effect of adding or subtracting 1SE of the log mean.

4. DISCUSSION

Using this system there is no demonstrable recruitment of a subpopulation of cells from the recirculating lymphocyte pool following injection of A2/1 blood. Furthermore there is no evidence that thoracic duct lymphocytes from donors injected with allogeneic blood have an increased capacity to produce a graft-versus-host reaction at any time following injection. Sprent, Miller and Mitchell (1971) found that thoracic duct lymphocytes from mice injected with F_1 hybrid spleen cells five days previously had increased graft-versus-host activity in similar F_1 hybrids. However as the present experiment did not include sampling at that time interval increased reactivity of the thoracic duct lymphocytes cannot be excluded. In addition slight latent variations of the graft-versus-host reaction cannot be excluded since no attempt was made to quantitate the reaction using varying doses of parental cells.

However in principle these findings are in contrast to those of Sprent, Miller and Mitchell (1971) who used mice and with Ford and Atkins (1971) but it is conceeded

that a very different system has been used. Ford and Atkins (1971) report that unpublished work of Gowans, Rowley, Ford, Atkins and Smith has demonstrated that in rats after intravenous injection of "several kinds" of antigen the recirculating lymphocyte pool does become specifically deficient in cells capable of mediating an immune response. Furthermore they also indicate that they have used F_1 hybrid cells as antigen in parental strain rats and have shown in this system some selection of antigen reactive cells.

As distinct from specific diminution of the graft-versus-host reaction occurring within 2 days of injection of antigen, there is general agreement that rats bearing enhanced renal or cardiac allografts over prolonged periods show normal graft-versus-host reactivity with donor strain antigen. Marquet, Heystek and Tinbergen (1971) demonstrated normal reactivity of lymphoid cells from enhanced rats bearing cardiac and renal allografts between 1 and 15 weeks after transplantation. Ockner, Guttman and Lindquist (1970b) showed normal reactivity of cells from enhanced rats bearing renal allografts 7 days after grafting, and French, Batchelor and Watts (1971) demonstrated normal

reactivity of cells from rats with enhanced renal allografts between 2 and 5 months after transplantation.

In summary this experiment does not demonstrate recruitment from the recirculating lymphocyte pool of cells reactive against donor antigen at any time following the initial conditioning injection with donor strain blood. It does however show that the conditioning injection does not induce a state of tolerance to the donor strain, and also that the prolongation of allograft survival induced by donor strain blood pre-treatment is not due to tolerance.

5. Evaluation of the ability of Hooded rats pre-treated with A2/1 blood and bearing long surviving cardiac allografts to reject donor strain split skin grafts

The experimental aim was to confirm that prolonged acceptance of cardiac allografts following injection with donor strain blood is not due to induction of specific tolerance.

Experimental Plan

5 recipient strain rats were each injected i.v. with

2 ml. donor strain blood 104 days before skin grafting, and cardiac allografts from the same donor strain were transplanted 14 days after the blood injections. At the time of applying split skin grafts the cardiac grafts were active.

Results

Table 15 shows that all skin grafts were rejected by the recipients in the time characteristic of control grafts (8 days).

6. DISCUSSION

This experiment demonstrates that rats pre-treated with donor strain blood and bearing long surviving cardiac allografts are fully capable of rejecting donor type skin grafts as quickly as untreated controls.

These findings are contrary to those of Marquet, Heystek and Tinbergen (1971) and of French, Batchelor and Watts (1971) who demonstrated that enhanced rats bearing long surviving organ allografts also accept donor type skin grafts for prolonged periods. Lucas, Markley and Travis (1970) and Morris and Lucas (1971)

Pre-Treatment	No. of Recipients	Skin Graft Survival (days)	
		Individual Values	Mean
NIL (From Anderson, James and Woodruff, 1967)	12	8,8,8,8,8, 8,8,8,8,8, 8,9	8
I.V. injection of donor strain blood Day -104. Cardiac allo-graft Day -90. Cardiac graft functioning at time of skin graft, i.e. on Day 0	5	8,8,8,8,8	8

Table 15: Survival time of A2/1 split thickness skin allografts on Hooded recipients of long surviving A2/1 cardiac allografts. Recipients pre-treated 14 days before cardiac transplantation with 2 ml. donor strain blood.

however found that rats bearing enhanced renal allografts rejected donor type skin as quickly as did controls.

The results of this and the preceeding experiment unequivocally demonstrate that the pre-treatment schedule used does not induce a state of tolerance to the donor antigens. The problem remains then to propose a mechanism by which the acceptance of a cardiac graft can be reconciled with rejection of a skin graft from the same source. The simplest explanation is that enhancement, perhaps on a waning scale, is responsible for cardiac graft survival but by 104 days has become inadequate for protection of skin grafts which are recognised to be difficult to enhance even under ideal conditions (Hutchin, 1966). Experiment 3, page 67, suggests that even cardiac grafts do not have prolonged survival when inserted 150 days after the enhancing injection and it is possible that the presence of a cardiac allograft may produce some self-perpetuating enhancement. Alternatively some form of graft adaptation as discussed on page 109 may reduce antigenicity of cardiac grafts which in the earlier stages were protected by strongly developed enhancement.

C H A P T E R XI

Evaluation of possible alteration of antigenicity of long surviving cardiac allografts

EVALUATION OF POSSIBLE ALTERATION OF ANTIGENICITY OF LONG SURVIVING CARDIAC ALLOGRAFTS

The subject of adaptation of allografts has been reviewed by Woodruff (1960), and it has been shown that allografts placed in the anterior chamber of the eye become less vulnerable to rejection with the passage of time (Woodruff and Woodruff, 1950). The mechanism of adaptation has not been elucidated, but the phenomenon may be of importance in clinical transplantation when, as sometimes happens, recipients of renal allografts require less immunosuppression as time passes.

1. Retransplantation of cardiac allografts

This experiment represents an attempt to assess possible reduction of antigenicity of long surviving allografts in "conditioned" recipients by retransplantation into otherwise untreated recipients.

Experimental Plan

Long surviving A2/1 cardiac allografts (in excess of 64 days) were removed from initial Hooded recipients which had been pre-treated i.v. with 2 ml. donor strain blood 14 days before operation, and were retransplanted

into secondary hosts. Heart No. 1 was retransplanted back into a donor strain (A2/1) animal. Nos. 2-6 were retransplanted into recipient strain (Hooded) animals. The graft survival time in the secondary hosts was assessed.

Results

These are summarised in Table 16.

Retransplantation of heart No. 1 back into the original donor strain (A2/1) resulted in survival of the graft until the recipient was sacrificed at 100 days. Marked variation in time of survival occurred in hearts 2-6 which were retransplanted to untreated recipient strain rats. The mean survival time of these hearts was 21.5 days. The standard error however amounted to 25% of the mean value. Those hearts which remained longest in conditioned recipients were rejected soonest in secondary recipients, while those which spent the shortest time in the conditioned recipients survived the longest in the secondary hosts.

Heart No.	Duration of Heart in Conditioned Host (days)	Strain of Secondary Host	Allograft Survival (days)	
			Individual Values	Mean ± SE
1	65	A2/1	100*	100
2	69	Hooded	35	
3	162	Hooded	10	
4	86	Hooded	25	21 ± 5.1
5	162	Hooded	9	
6	90	Hooded	28	

Table 16: Cardiac allograft survival. Effect of retransplantation of long surviving A2/1 cardiac allografts from initial recipient strain (Hooded) animals (conditioned on day -14 before transplantation with 2 ml. donor blood i.v.) into untreated secondary hosts.

2. DISCUSSION

The principal defect of this experiment is the small number of constituent animals. Retransplantation of hearts is technically difficult and is attended by a high mortality. However it is evident that the antigenicity of hearts with prolonged survival in conditioned allogeneic hosts is not qualitatively altered, as heart No. 1 survived in the final recipient of donor strain until termination of the experiment at 100 days. Hearts Nos. 2-6 showed wide individual variation of survival time and the difference between the mean survival of these hearts (21 ± 5.1 days) and that of controls in Table 4 (14 ± 1.3 days) is not statistically significant ($P < 0.25$). However it is of interest that hearts Nos. 3 and 5 in Table 16 which were rejected soonest (10 days and 9 days) had resided the longest in the intermediate hosts (162 days).

Conversely heart No. 2 which remained for the shortest time in the intermediate host (65 days) had the longest ultimate survival time.

Clearly conclusions drawn from this aspect of the experiment must be guarded in view of the small number

of constituent animals. However it seems possible that some hearts retransplanted from "conditioned" intermediate hosts may have some quantitative alteration in antigenicity.

Others have demonstrated a similar effect with rat renal allografts. Ockner, Guttman and Lindquist (1970b) using an enhancement model showed that "when several long surviving LBN F_1 allografts were retransplanted into normal L recipients, rejection did not occur normally". Marquet, Heystek and Tinbergen (1971) also noted prolonged survival of a single rat which was the recipient of a renal allograft which had been enhanced for 1 year in an allogeneic intermediate recipient.

The mechanism of prolonged survival following retransplantation is open to conjecture, but antibody coating of antigenic sites, cellular substitution, and loss of certain antigenic cells require consideration. It seems inconceivable that "coating" antibody could persist long enough to give prolonged graft survival, though continuous endogenous production of antibody with consequent graft protection seems possible. Unlike skin grafts cardiac grafts could hardly be replaced by "creeping substitution" of constituent

cells. The third possibility remains.

Recently much interest has centred round the role of the "passenger leucocyte" within grafts, and the subject has been reviewed by Billingham (1971). Several studies suggest that much of the antigenicity of a graft is related to the content of passenger leucocytes lying in the capillary bed and tissue spaces of the organ. Ockner, Guttman and Lindquist (1970b) have suggested that the mechanism of enhancing antibody in prolonging the lives of certain types of allografts may depend on its capacity to destroy passenger leucocytes. Several experiments have recently been reported which emphasise the importance of these cells. Guttman, Lindquist and Ockner (1969) have shown that renal isografts that carry allogeneic passenger cells develop signs of rejection after transplantation. Conversely it was found that abrogation of rejection of rat renal allografts could be obtained by replacement of the prospective donor's haemopoietic cells with cells isologous with the future host. Guttman, Carpenter, Lindquist and Merrill (1967) also noted that renal allografts from donors treated with ALS are not rejected by normal rats as quickly as kidneys from untreated donors, which suggests a partial

elimination of ALS susceptible antigenic cells from the grafts.

Recently Freeman, Chamberlain, Reemtsma and Steinmuller (1971) have demonstrated the importance of passenger cells in rat cardiac allografts by treatment of prospective donors with agents designed to produce leucopoenia which resulted in prolongation of survival time of the grafts.

It is probable therefore that allografts do owe part of their antigenicity to passenger cells and that elimination of such cells may prolong allograft survival. It seems unlikely however that grafts could be made non-antigenic even by total elimination of all passengers, since presumably muscle and other cells also carry antigen determinants. Late rejection crises of human renal allografts occurring some years after transplantation are well recognised, and it seems inconceivable that persisting passenger cells of donor origin could be the instigators of such events.

An alternative way in which cells may lose antigenicity is by "antigenic modulation". Old, Stockert, Boyse and Kim (1968) demonstrated loss of the TL antigen by mouse thymocytes through the action of specific

antibody. Coating of antigen with antibody was excluded. However no evidence has yet been produced to show that modulation occurs in homografts.

CONCLUSION

CONCLUSION

The experiments in this thesis have shown that pre-treatment of recipients of cardiac allografts with donor strain blood, thoracic duct lymphocytes, and spleen cells induces marked immunologically specific prolongation of graft survival time. It appears that a further injection of donor blood after transplantation may produce additional prolongation of graft survival. Using spleen cells the optimum conditions for induction of the phenomenon were demonstrated, and a four-fold increase in survival time was achieved. It is of interest that in no case did injection of recipients with donor strain cells before or after transplantation induce accelerated graft rejection.

The mechanism of the effect has not been proved conclusively, but the optimum pre-treatment dose, timing, and route of administration are suggestive of an enhancing regime. Demonstration of 7S antibodies in response to donor strain antigen furthers this suggestion since enhancing antibodies are known to be of this class. Although such antibodies have been shown to occur following pre-treatment of recipients their causal

relationship to prolongation of graft survival time is more difficult to assess, and this point has not been precisely defined in the present study.

It is of interest that passive transfer of serum from pre-treated rats to recipients of cardiac allografts did not prolong graft survival. At first sight this seems to suggest that serum factors may not be responsible for abrogation of rejection in the earlier experiments. However it is possible that the optimum dose of serum was not given, or that hyperimmunisation of the serum donor may be necessary to raise an effective serum. Others have raised effective sera using three spaced injections of 10^8 donor strain cells, the first accompanied by Freund's adjuvant (Stuart, Saitoh and Fitch, 1968; Lucas, Markley and Travis, 1970). As well as this in most studies F_1 hybrid graft donors have been used which reduces the degree of antigenic disparity between donor and recipient.

It is evident that the pre-treatment schedule of prospective cardiac allograft recipients does not induce immunological tolerance since thoracic duct lymphocytes of such rats had normal graft-versus-host reactivity in F_1 hybrid animals. In addition evidence of some degree

of rejection was found in all heart transplants removed from pre-treated animals, which suggests that such rats recognise and react against the foreign antigen. The ability of pre-treated rats carrying long surviving cardiac allografts to reject donor type skin grafts further indicates that such rats are not immunologically tolerant.

Application of enhancement to human recipients of allografts is an attractive prospect since the immunosuppression is specific and its use would not entail the complications produced by agents which act non-specifically.

Before considering the use of enhancement clinically experiments using a variety of different species are of obvious interest. In general most rodents appear to exhibit enhancement though until recently it was thought that the Syrian Hamster was an exception in view of failure to raise sera with cytotoxic or haemagglutinating properties from these animals (Billingham and Silvers, 1964a; Palm, Silvers and Billingham, 1967). It was deduced that the hamsters were unable to form cytotoxic antibodies in response to skin allografts (Billingham and Silvers, 1964b; Shaffer, 1969). However Ramseier

and Lindemann (1971) have produced evidence that the test system used in these studies was inadequate. Efforts to enhance canine renal allografts have met with varied success. Wilson, Rippin, Dagher, Kinneart and Busch (1969) and Holl-Allen (1971) showed that protection of such grafts could be achieved by a combination of donor cell pre-treatment and minimal doses of immunosuppressive drugs.

It thus appears that at least some experimental animals do display the phenomenon of enhancement though to varying degrees, and it is possible that man is not an exception. However it would be unwise to presume that allografts in the rat and in man are affected in the same way by preformed antibodies. The apparent impossibility of inducing hyperacute rejection of rat cardiac allografts suggests that this is not so. Chavez-Peon, Monchik, Winn and Russell (1971) have suggested that the rat model may not be an appropriate one for clinical studies, and have also shown that renal allografts in rats which have high levels of circulating alloantibodies are not subjected to very early and acute destruction. They also demonstrated however that the rat is capable of mounting reactions which are

responsible for hyperacute rejection by the use of xenografts. In addition Kissmeyer-Nielsen, Olsen, Petersen and Fjeldborg (1966) and others have pointed out that in man the presence of preformed antibody to donor cells in recipients of renal allografts ensures hyperacute graft rejection.

A technique which avoids the damaging effects of antibody on graft survival has been used by Batchelor, French, Cameron, Ellis, Bewick and Ogg (1970) to passively enhance a renal allograft in a child. The patient received non-complement binding antibody fragments derived from serum prepared by immunising the father with leucocytes from the mother who was also the graft donor. It was found that no further immunosuppression was required for three months. A further approach might lie in making antibody preparations non-cytotoxic by separating out enhancing from cytotoxic portions (Russell, 1971).

It seems reasonable to speculate that passive enhancement will come to be used clinically as a means of combating rejection, possibly in conjunction with standard immunosuppressive agents. In this way it may be possible to reduce the dose of drugs to a level at

which side effects are negligible.

Protection of allografts in man by pre-treatment of recipients with donor cells is a less likely eventuality in view of the reported effect of cytotoxic antibody on human allograft survival. If however the technique does prove ultimately to have clinical potential the inherent advantages are obvious. The present study has shown that donor cells exert a protective effect on rat allografts when given as late as during transplantation itself. If this is true in man also, it would be possible to harvest the cells and graft simultaneously. The method could then be used when as frequently happens organ donors present with little prior warning.

Specific immunosuppression provides a new and potentially rewarding approach to the problem of rejection in clinical transplantation. It is not suggested that it will replace conventional immunosuppressive techniques in the foreseeable future, but it is to be hoped that at least it may prove a valuable adjunct to such treatment. At best there is the possibility that it may ultimately emerge as a primary defence against rejection.

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